

**PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Yinghui Dan *et al*

Serial No.: 10/064,001

Filed: June 3, 2002

FOR: A NOVEL METHOD FOR THE  
PRODUCTION OF TRANSGENIC  
PLANTS

Group Art Unit: 1638

Examiner: Keith O. Robinson

Atty. Dkt. No.: MONS:130US

**BRIEF ON APPEAL**

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Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit this Appeal Brief. The date for filing this Brief is August 5, 2011 in view of the accompanying request for extension of time and fees. The fee for filing this Brief is being concurrently filed. Should any additional fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, or should an overpayment be made, the Commissioner is authorized to deduct or credit said fees from or to SNR Denton LLP, Deposit Account No. 19-3140/11000023-2229/MONS:130US.

## **I. REAL PARTY IN INTEREST**

The Real Party in Interest is Monsanto Company, the parent company of assignee Monsanto Technology LLC.

## **II. RELATED APPEALS AND INTERFERENCES**

There are no related appeals or interferences

## **III. STATUS OF THE CLAIMS**

Claims 1-16 were originally filed with the Application. Claims 1-7 and 9-16 were amended during prosecution and claim 8 was canceled. Claims 1-7 and 9-16 are therefore now pending and the subject of this appeal. A copy of the appealed claims as they currently stand is provided in Section VIII.

## **IV. STATUS OF AMENDMENTS**

Claim amendments were filed in the case on August 22, 2005; May 17, 2006; December 11, 2006; October 27, 2008; and November 3, 2009. All of the amendments have been entered by the examiner. No amendments were made subsequent to the Final Office Action.

## **V. SUMMARY OF CLAIMED SUBJECT MATTER**

The subject matter defined in independent claim 1 concerns a method of producing multiple transgenic wheat plants from a single wheat mesocotyl explant, comprising providing a wheat mesocotyl explant presenting a plurality of meristems; culturing the explant in a first medium to induce production of a plurality of buds; introducing exogenous DNA into more than one of such buds; transferring the buds to a second medium for elongation of the buds into shoots; harvesting and transferring the

shoots to a culture medium for root development; and culturing the shoots to produce multiple transgenic wheat plants. Specification at p.1-2, ¶ [0003]; p. 6, ¶ [0031]; p. 4, ¶ [0028]; and p. 21, ¶ [0081].

The subject matter defined in independent claim 13 concerns a method of producing multiple transgenic wheat plants from a single explant comprising: providing a wheat mesocotyl explant presenting a plurality of meristems; culturing the explant in a first medium comprising the recited levels of thidiazuron and 2,4-D to induce production of a plurality of buds; introducing exogenous DNA into at least one of the cells of such buds; removing the buds from the first medium and transferring the buds to a second medium for elongation of the buds into shoots; and culturing the shoots to produce multiple transgenic wheat plants. Specification at p.1-2, ¶ [0003]; p. 6, ¶ [0031]; p. 4, ¶ [0028]; and at p. 21, ¶ [0081].

## **VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

Were claims 1-7 and 9-16 properly rejected under 35 U.S.C. §103(a) as being unpatentable over Zhong *et al.* (*Planta* 187:483-489, 1992), in view of Bowen *et al.* (U.S. Patent 5,736,369), Cheng *et al.* (*Plant Physiol.* 115:971-980, 1997), Bartok *et al.*, (*Pl. Cell Tiss. Org. Cult.* 22:37-41, 1990), and Weeks *et al.* (*Plant Physiol.* 102:1077-1084, 1993)?

## **VII. ARGUMENT**

### **A. The Cited References Do Not Render the Claims Obvious**

The Examiner rejects claims 1-7 and 9-16 under 35 U.S.C. §103(a) as being obvious over Zhong *et al.* (*Planta* 187:483-489, 1992; **Exhibit A**), in view of Bowen *et al.* (U.S. Patent 5,736,369; **Exhibit B**); Cheng *et al.* (*Plant Physiol.* 115:971-980, 1997;

**Exhibit C**); Bartok *et al.*, (*Pl. Cell Tiss. Org. Cult.* 22:37-41, 1990; **Exhibit D**), and Weeks *et al.* (*Plant Physiol.* 102:1077-1084, 1993; **Exhibit E**). Claim 1 reads as follows:

1. A method of producing multiple transgenic wheat plants from a single explant comprising:

providing an **explant presenting a plurality of meristems**;

culturing said explant in a first **multiple bud inducing media** suitable for inducing production of a plurality of buds from at least one of said meristems;

**introducing exogenous DNA** into more than one of said plurality of buds;

removing said plurality of buds from said first media and transferring said plurality of buds to a second media suitable for induction of elongation of said buds into shoots;

harvesting and transferring said shoots to a culture medium that promotes root development; and

culturing said transferred shoots to produce multiple transgenic wheat plants **wherein the explant is a wheat mesocotyl explant.**

(emphasis added). Independent claim 13 contains similar limitations to claim 1, but also recites use of the phytohormones thidiazuron and 2,4-D. All of the appealed claims depend from claim 1 or claim 13. The highlighted portions of claim 1 shown above are limitations asserted to be taught by cited references.

The primary reference relied upon by the Examiner, Zhong, is asserted to teach use of an explant with a plurality of meristems, and use of a multiple bud-inducing medium, but is conceded to not teach use of (a) wheat mesocotyl explants; (b) introducing exogenous DNA; (c) selecting for plants with resistance to a selective agent; and (d) introducing exogenous DNA via *Agrobacterium*. Bowen is cited for teaching introduction of exogenous DNA into a plurality of meristems. Bartok is cited for teaching use of a wheat mesocotyl explant. Limitations relating to resistance to a selective agent (asserted to be taught by Weeks) and introduction of exogenous DNA via *Agrobacterium* (asserted to be taught by Cheng) are found in dependent claims, such as

claims 9 and 12. The Examiner alleges that one of skill in the art would be motivated to combine the cited references to arrive at the invention and thus that the claims are obvious. The combination of these references, however, does not render the claims obvious as is explained below. Reversal is thus respectfully requested.

**1. Zhong and Bartók References are Not Properly Combined.**

As explained above, Zhong is principally relied upon, and together with Bartók, is critical to the rejection. The Examiner has conceded that Zhong does not teach use of wheat mesocotyl explants, and uses Bartók to cure this defect. The secondary reference of Bartók *et al.* is thus needed for teaching limitations of claim 1 relating to culturing a wheat mesocotyl explant, as Bartok is the only reference cited with respect to use of this explant. However, as explained below, Zhong and Bartok are contradictory and not properly combined in an attempt to establish obviousness whether alone or together with the knowledge in the art and other cited references.

**a. The explants of Zhong and Bartók are distinct, and their teachings and those of Bowen regarding explant preparation would not be considered combinable by one of skill in the art.**

The teachings of Zhong and Bartok regarding the preparation of explant tissues and the structures they would contain are contradictory and one of skill in the art would not be motivated to try to combine them. This is true not only with respect to their sources (*i.e.* corn vs. wheat), but also in view of the specific teachings of Zhong and Bartok relating to isolation of their explants and subsequent tissue culture conditions. In particular, Zhong utilizes shoot tips that are **separated from the mesocotyl**, while Bartok uses mesocotyl tissues isolated away from the coleoptile and shoot apex (tip). Thus the explants of Bartók and Zhong are clearly distinct even before tissue culture commences.

Appellants point to Fig. 19-7 in **Exhibit F** (formerly Exhibit 1 of the Appeal Brief filed in the present case on February 19, 2008: pp. 383-385 from Salisbury & Ross, Eds., *Plant Physiology*, 3<sup>rd</sup> Ed., Wadsworth Publ., 1988). This figure illustrates a maize seedling, also generally representative of other monocots, showing the location of the mesocotyl and other structures in a maize seedling.

Bartók prepares their wheat mesocotyl explant by cutting so that “...most of the germ tissues [*i.e.* germ being typically defined as both the embryo and scutellum fractions of the wheat kernel] were removed by means of four cuts with a scalpel, so that only mesocotyl [*i.e.* internode] remained on the scutellum” (page 38, left column). Indeed, Bartók only germinates their wheat seed for 26 hours, and the stem has not yet started to elongate when the germ is removed from the seed (see Fig. 1A). Thus Bartók teaches explant preparation by cutting their mesocotyl explant away from the coleoptilar node to which the coleoptile and shoot apex are attached, and **removing the shoot apex** among other structures even before the stem has elongated.

In contrast, Zhong’s corn shoot tips are derived from 1 week old elongating (3-5 cm in length) corn seedlings. Zhong explicitly and repeatedly teaches the use of **shoot tips** (with nearby portions of leaf, stem, and primordia) as their explant source. For instance, the location of Zhong’s cut is made by identifying the localized enlargement of the seedling at the junction between the mesocotyl and coleoptile (Zhong, page 483, right column, bottom), and apparently then cutting their explant away from the mesocotyl. Zhong’s explants would thus contain the shoot tips with nearby portions of leaf, stem, and leaf primordia, and lack the mesocotyl which is only mentioned in order to define the location of their cut.

These references therefore teach use of distinct explants, and a skilled worker would not simply attempt to combine these references as asserted by the Examiner, and would have no reasonable expectation that this could even be done. Absent hindsight reasoning, a skilled worker would not even know, in view of the disparate teachings of these references, which of the many possible variables in explant preparation might be important, or how to combine various aspects of the teachings of Zhong with aspects of the teachings of Bartok. Indeed the contradictory teachings of these two references with respect to explant preparation are not even acknowledged by the Examiner. These teachings for explant preparation are further distinct from those of the present Application, which teaches that the mesocotyl explant should include several different primary meristems *i.e.* an apical meristem and axillary meristems (see Specification ¶¶29-30). A skilled worker would simply not consider the teachings combinable in the absence of additional information or experimentation, which would not be routine.

Bowen is not asserted with respect to explant preparation and use, and does not teach isolation of an explant prior to transformation. Although Bowen is cited to teach the possible excision of an already transformed shoot meristem at col. 7, l. 55, such a meristem would be isolated by cutting as in Fig. 2, to remove the shoot apex. Thus Bowen does not cure the defect in Zhong, either alone or in combination with Bartok, and instead points to yet another different approach for obtaining transformed plants. The remaining references are further not asserted to teach the elements of claim 1 or 13. The obviousness rejection is therefore not properly supported and its reversal is respectfully requested.

**b. The tissue culture conditions of Zhong and Bartók are distinct.**

Zhong and Bartók are also contradictory regarding the tissue culture conditions they utilize, and thus one of skill in the art would further not attempt to combine the references or even believe that they could be with any reasonable expectation of success. For instance, in order to produce meristematic tissues, Bartók utilizes induction of **organogenic callus** which occurs on the remaining mesocotyl tissues attached to the scutellum, to allow for *de novo* formation of additional meristematic tissue (*e.g.* see Bartók, Fig. 2; see also page 40, right column). In contrast, the only callus induction described by Zhong is **embryogenic callus**, arising on the shoot tip, and most multiple shoot cultures (“MSC”) arose by activation of axillary buds without callus formation (Zhong, page 486, left column; and Figs. 3 and 10). Bartók’s approach and the required culture conditions are thus distinct from those of Zhong, especially since organogenic and embryogenic calli are well known in the art to be developmentally distinct and the conditions needed for induction of one or the other are also typically distinct. Again, a skilled worker would not know which of the many possible variables in tissue culture conditions might be important, or how to combine various aspects of the teachings of Zhong with aspects of the teachings of Bartók.

This is further demonstrated in that the initial tissue culture medium of Zhong contains **cytokinin but no auxin**, while medium of Bartók contains **auxin but no cytokinin**. Further, the only medium of Zhong that contains no cytokinin (*e.g.* BA) is medium “F” which is only used for rooting of developed shoots and not for production of shoot meristems or buds. Bartók does not even discuss use of cytokinin in any medium. Instead Bartók states that “only mature seeds and 2,4-D [*i.e.* auxin] are required for callus induction in wheat” [page 37, right column]. Since these two references utilize different

tissue culture conditions on distinct explant tissue leading to distinct developmental pathways, a skilled worker would not consider them combinable in the absence of additional information or experimentation, which would not be routine. In view of this, reversal of the rejection is respectfully requested.

**c. Additional references do not cure the defect in combinability.**

As established above, Bartók is therefore not properly combined with Zhong because the proposed modification or combination would change the principle of operation of Zhong. M.P.E.P. 2143.01 VI. That is, the principle of operation of Zhong is clearly different from that of Bartók with respect to at least:

- (1) The explant which is utilized (shoot tip vs. mesocotyl lacking shoot tip);
- (2) The overall tissue culture approach utilized (*i.e.* application of plant hormones to yield direct organogenesis via “multiple shoot clump” (MSC) production, or embryogenic callus for Zhong; but organogenic callus for Bartók, and
- (3) The hormone composition of the tissue culture media (presence of cytokinin or auxin).

Zhong and Bartok teach away from each other with respect to the preparation of their explants and their subsequent tissue culture conditions. Thus, one of skill in the art would not have been motivated to attempt to combine Zhong and Bartok, and would not have had any reasonable expectation of success in doing so. Since the combination of Zhong and Bartok is critical for supporting the rejection, the rejection fails. The additional references by Bowen, Cheng, and Weeks are not asserted to, nor do they, cure the defects in Zhong, or in Zhong in view of Bartok, as relating to explant choice and preparation, and subsequent tissue culture parameters. Again, Bowen is only asserted

with respect to introduction of exogenous DNA, and not with respect to source or preparation of explant tissue, or tissue culture conditions. The limited teachings in Bowen relating to tissue excision (*e.g.* col. 7) are only after transformation has occurred, and result in removal of the shoot apex, thus contradicting Zhong. Cheng and Weeks are not even asserted with respect to limitations in claim 1, instead only with respect to limitations in dependent claims. Thus Cheng and Weeks are not relevant to claims 1 and 13, and can not render a claim dependent on either of these claims as obvious. The rejection is therefore not properly supported, and its reversal is therefore respectfully requested

**2. The cited references teach away from the claimed invention.**

As noted at ¶30 of the present specification, “[i]t is important when excising the mesocotyl explant to leave a whole piece of the scutellum attached to the explant, in order to avoid damaging the meristem tissue of the scutellar node” [emphasis added]. In contrast, Zhong teaches excising the shoot tip explant away from the mesocotyl, as well as the scutellum, when they teach preparation of a shoot tip explant by locating the junction of mesocotyl and coleoptile (*e.g.* page 483, Materials and Methods section). Bartók does not cure this defect, and if anything also teaches away, because they teach removing most of the germ (*i.e.* embryo) from their explant, leaving only mesocotyl attached to the scutellum. Thus, Bartók’s explant would lack the “several different and distinct primary meristems, such as...coleoptile node, a first leaf bud...leaf nodes...” [Specification, at ¶30]. Although Bartók apparently attempts to overcome the loss of such primary meristems by forming secondary meristems via callus culture, this yet again demonstrates the distinct approaches taken by Zhong and Bartok (and the present

invention). Bowen is only asserted with respect to introduction of exogenous DNA, and not with respect to source or preparation of explant tissue; and in any event, to the extent that Bowen teaches excision of tissues, this results in removal of shoot axillary meristems from the shoot apex. Cheng and Weeks are not even asserted with respect to any limitations in claim 1, instead only with respect to limitations in dependent claims. Accordingly, reversal of the rejection is respectfully requested.

**B. No Expectation of Success is Provided.**

The Examiner maintains the assertion that a skilled worker would have an expectation of success in combining Zhong, Bowen, Bartók, Weeks, and Cheng to yield the invention. A review of the references and knowledge in the art as of the invention reveals, however, that the assertion of an expectation of success is not supported and is in fact unsupportable.

**1. The rejection is without evidentiary support.**

Factual findings and conclusions of an examiner must be supported by “substantial evidence” in accordance with the Administrative Procedure Act (APA). *In re Gartside*, 203 F.3d 1305, 1315, 53 USPQ2d 1769, 1775 (Fed. Cir. 2000). Here, the Examiner’s conclusions are lacking any such evidence. As noted above, the mesocotyl explant of Bartók, lacking a shoot tip, is not equivalent to the shoot tip explant of Zhong, since Bartók removes the shoot tip from their mesocotyl explant, while Zhong removes the mesocotyl from their shoot tip explant. Additionally, Bartók teaches organogenic callus induction from their mesocotyl explant, while Zhong describes further shoot formation, or embryogenic callus formation leading to later shoot development, from their shoot tip explant. These distinct approaches are achieved via use of different tissue culture parameters, as is conceded in the final Action at page 11. Given these differences

a skilled worker would not have any reasonable expectation of combining the teachings while expecting success, particularly give the high degree of unpredictability in the art.

Further, claims 1 and 13 recite use of a wheat mesocotyl explant. The present Specification, for instance at ¶¶29-30, defines mesocotyl explants as including several primary meristems including scutellar node, coleoptile node, first leaf bud, and an apical meristem, such that the explant comprises an apical meristem and axillary meristems. However, the explants of Zhong and Bartok are both clearly distinct from this, as well as from each other. As noted above, Zhong's explant is prepared by excising their shoot tip explant away from the mesocotyl as well as the scutellum. Bartok's explant is prepared by removing most of the germ (*i.e.* embryo) from their explant, leaving only mesocotyl (internode) attached to the scutellum, and not utilizing the shoot apex. Neither of these two references describe preparation and use of a mesocotyl explant as presently defined.

The additional references by Bowen, Cheng, and Weeks, do not cure these defects in Zhong and Bartok, or in Zhong, absent Bartok. Bowen does not teach preparation of a meristem explant, *e.g.* a portion of an embryo or plant, prior to transformation. Bowen is only asserted with respect to introducing exogenous DNA into a plurality of meristems. Appellants' review of Bowen further shows, for instance at col. 3, line 63, to col. 6, line 15, that Bowen targets the apical meristem found in the developing apical dome as the site for introducing exogenous DNA, followed by optional meristem reorganization (*e.g.* col. 7, line 18 and following). Axillary buds or other meristems (*e.g.* at col. 8, lines 36-50) are only discussed as being induced or reorganized after transformation has occurred, and are used instead of the apical meristem, after wounding the apical meristem or otherwise suppressing its development. Thus a skilled worker would not combine Bowen

with Zhong, or with Zhong and Bartok to yield any expectation of success in reaching the presently claimed invention. Cheng and Weeks are not asserted as relating to limitations in claims 1 and 13. The rejection is therefore not properly supported.

There would further have been no reasonable expectations as to what the developmental response would be, for instance, of the mesocotyl explant of Bartók when placed under tissue culture conditions of Zhong, as suggested by the Examiner. Bartok's explant, with most of its primary meristems, primordia, and buds having been removed, would not be expected to efficiently or rapidly yield a multiple shoot culture or rapidly yield a large number of adventitious shoots, since removal of leaf primordia and stem tissue which would include bud primordia, as taught by Bartok, would interfere with or delay the shoot formation desired by Zhong. Given the disparate and conflicting teachings of these references, they simply would not have provided the necessary reasonable expectation of success needed to support the rejection. This fatal flaw is not remedied by Cheng and Weeks, which are not cited with respect to limitations in claims 1 and 13 and are only cited with respect to dependent claim limitations. Appellants therefore respectfully request that the rejection be reversed.

## **2. The rejection relies upon impermissible hindsight.**

The Examiner has failed to establish in the cited references or art in general the necessary motivation and reasonable expectation of success in arriving at the invention, and thus is relying upon impermissible hindsight in combining the cited reference to formulate the rejection. The Federal Circuit in *In re Kubin* stated, for example that:

[t]o differentiate between proper and improper applications of 'obvious to try,' this court outlined two classes of situations where **'obvious to try' is erroneously equated with obviousness under § 103**. In the first class of cases,

what would have been ‘obvious to try’ would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. [*In re O’Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988)]

*Id.* In such circumstances, where a defendant **merely throws metaphorical darts at a board filled with combinatorial prior art possibilities**, courts should not succumb to hindsight claims of obviousness.”

561 F.3d 1351 (Fed. Cir. 2009) (emphasis added).

Here, the Examiner’s assertions represent just such hindsight reasoning cautioned against, since the cited art gives no indication as to which explant preparation and tissue culture parameters could be chosen and used with success from amongst a nearly endless different number of combinations. The teachings of Zhong, Bowen, and Bartók are contradictory with respect to the choice of target tissue for transformation; explant preparation; which meristems might be present at transformation or later induced; whether callus culture is to be utilized, and if so, if organogenic or embryogenic approaches should be used. Zhong and Bartok are further contradictory with respect to tissue culture conditions for generating callus and/or shoots, and Bowen apparently attempts to avoid isolated callus culture altogether, instead trying to reorganize meristems and produce transformed shoots. A skilled worker could therefore combine the teachings of these references in a nearly limitless number of ways, with no direction toward the present invention being found in the art. This is underscored by the contradictory nature of the teachings. As such, the rejection relies upon precisely the type of rationale prohibited by the Federal Circuit in *In re Kubin* and should be reversed as such.

**C. Conclusion.**

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

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## **VIII. CLAIMS APPENDIX**

### **APPEALED CLAIMS:**

1. A method of producing multiple transgenic wheat plants from a single explant comprising:

providing an explant presenting a plurality of meristems;

culturing said explant in a first multiple bud inducing media suitable for inducing production of a plurality of buds from at least one of said meristems;

introducing exogenous DNA into more than one of said plurality of buds;

removing said plurality of buds from said first media and transferring said plurality of buds to a second media suitable for induction of elongation of said buds into shoots;

harvesting and transferring said shoots to a culture medium that promotes root development; and

culturing said transferred shoots to produce multiple transgenic wheat plants wherein the explant is a wheat mesocotyl explant.

2. The method of claim 1, wherein said multiple bud inducing media comprises a cytokinin and an auxin.

3. The method of claim 2, wherein said cytokinin is thidiazuron.

4. The method of claim 2, wherein the concentration of said cytokinin is between 2.0mg/L and 7.5mg/L.

5. The method of claim 2, wherein said cytokinin is thidiazuron and said auxin is selected from the group consisting of 2,4-D and picloram.

6. The method of claim 5, wherein the concentration of thidiazuron is between 2.0mg/L and 7.5mg/L and the concentration of auxin is between 0.5mg/L and 2.0mg/L.

7. The method of claim 1, wherein said plurality of meristems contains the scutellar node.

9. The method of claim 1, wherein said exogenous DNA comprises a nucleic acid encoding a protein capable of conferring resistance to a selection agent.
10. The method of claim 9, further comprising a step of selecting for plants containing the protein conferring resistance to a selection agent.
11. The method of claim 1, wherein said exogenous DNA is introduced via biolistic particle bombardment.
12. The method of claim 1, wherein said exogenous DNA is introduced via *Agrobacterium*-mediated transformation.
13. A method of producing multiple transgenic wheat plants from a single explant comprising:
- providing a wheat mesocotyl explant presenting a plurality of meristems;
  - culturing said wheat mesocotyl explant on a first media, comprising thidiazuron at a concentration of between about 2.0 mg/L and 7.5 mg/L, and 2,4-D at a concentration of about 0.5 mg/L and 2.0 mg/L, to induce the production of a plurality of buds from at least one of said plurality of meristems;
  - introducing exogenous DNA into at least one of the cells of said plurality of buds;
  - removing said plurality of buds from said first media and transferring said plurality of buds to a second media suitable for induction of elongation of said buds into shoots;
  - culturing said shoots to produce multiple transgenic wheat plants.
14. The method of claim 13, wherein said exogenous DNA is introduced via *Agrobacterium*-mediated transformation.
15. The method of claim 13, wherein said exogenous DNA is introduced via biolistic particle bombardment.

16. The method of claim 13, further comprising a step of selecting for plants containing the exogenous DNA.

## IX. EVIDENCE APPENDIX

- Exhibit A:** Zhong *et al.*, *Planta* 187:483-489, 1992; reference “U” as cited by the Examiner in the list of August 3, 2009.
- Exhibit B:** Bowen *et al.* (U.S. Patent 5,736,369); reference “A” as cited by the Examiner in the list of August 3, 2009.
- Exhibit C:** Cheng *et al.* (*Plant Physiol.* 115:971-980, 1997); reference “V” as cited by the Examiner in the list of August 3, 2009.
- Exhibit D:** Bartok *et al.* *Plant Cell Tissue and Organ Culture* 22:37-41, 1990; reference “W” as cited by the Examiner in the list of August 3, 2009.
- Exhibit E:** Weeks *et al.* (*Plant Physiol.* 102:1077-1084, 1993); reference “X” as cited by the Examiner in the list of August 3, 2009.
- Exhibit F:** pp. 383-385 from Salisbury & Ross, Eds., *Plant Physiology*, 3<sup>rd</sup> Ed., Wadsworth Publ., 1988; formerly provided as Exhibit 1 in the Appeal Brief filed on February 19, 2008.

**X.     RELATED PROCEEDINGS APPENDIX**

None.

## **EXHIBIT A**

# In-vitro morphogenesis of corn (*Zea mays* L.)

## I. Differentiation of multiple shoot clumps and somatic embryos from shoot tips

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Received 19 June 1991; accepted 23 March 1992

**Abstract.** In-vitro methods have been developed to regenerate clumps of multiple shoots and somatic embryos at high frequency from shoot tips of aseptically-grown seedlings as well as from shoot apices of precociously-germinated immature zygotic embryos of corn (*Zea mays* L.). About 500 shoots were produced from a shoot tip after eight weeks of culture (primary culture and one subculture of four weeks) in darkness on Murashige and Skoog basal medium (MS) supplemented with 500 mg/L casein hydrolysate (CH) and 9  $\mu$ M N<sup>6</sup>-benzyladenine (BA). In this medium, shoots formed in shoot tips as tightly packed "multiple shoot clumps" (MSC), which were composed of some axillary shoots and many adventitious shoots. When the shoot tips were cultured on MS medium containing 500 mg/L CH, 9  $\mu$ M BA and 2.25  $\mu$ M 2,4-dichlorophenoxyacetic acid (2,4-D), most of the shoots in the clumps were adventitious in origin. Similar shoot tips cultured on MS medium containing 500 mg/L CH, 4.5  $\mu$ M BA and 2.25  $\mu$ M 2,4-D regenerated many somatic embryos within eight weeks of culture. Somatic embryos were produced either directly from the shoot apical meristems or from calli derived from the shoots apices. Both the MSC and the embryos produced normal shoots on MS medium containing 2.25  $\mu$ M BA and 1.8  $\mu$ M indole-3-butyric acid (IBA). These shoots were rooted on MS medium containing 3.6  $\mu$ M IBA, and fertile corn plants were grown in the greenhouse. The sweet-corn genotype, Honey N Pearl, was used for the experiments described above, but shoot-tip cultures from all of 19 other corn genotypes tested also formed MSC on MS medium containing 500 mg/L CH and 9  $\mu$ M BA.

**Key words:** Shoot clump – Shoot meristem – Shoot organogenesis – Somatic embryogenesis in vitro – *Zea* (shoot and embryo formation in vitro)

### Introduction

An efficient and reproducible in-vitro-regeneration technique permitting the production of whole plants from transformed cells is an integral part of successful genetic engineering of plants (Potrykus 1990; Vasil 1990). Currently, immature zygotic embryos are the most reliable explant source to develop regeneratively-competent calli, cell suspensions and protoplasts for transformation of corn (Rhodes et al. 1988; Fromm et al. 1990; Gordon-Kamm et al. 1990). In our attempts to identify an alternative and highly-regenerative explant source, we have succeeded in developing several in-vitro procedures that permit one to obtain high-frequency differentiation of adventitious shoots and somatic embryos in corn-shoot tips excised from seedlings grown in vitro.

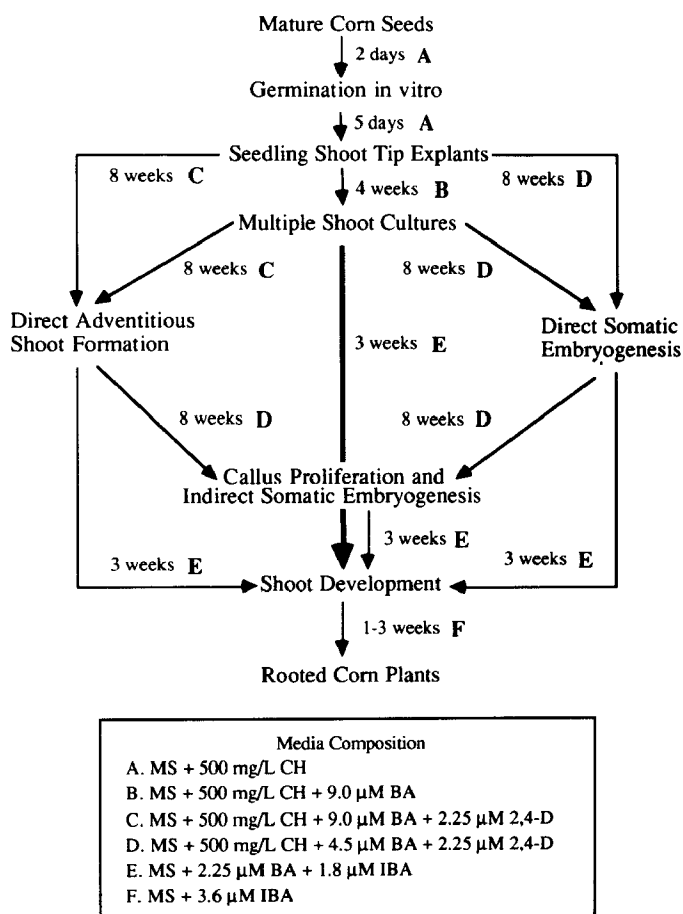
### Materials and methods

Mature seeds (caryopses, kernels) of twenty corn (*Zea mays* L.) genotypes listed in Table 1 were used for this research. The seeds were surface-sterilized first in 70% ethanol for 10 min, washed once with sterile water and then soaked for 30 min in 2.6% sodium hypochlorite (prepared from commercial bleach) containing 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate). The surface-sterilized seeds were washed three times with sterile distilled water and germinated aseptically on Murashige and Skoog (1962) basal medium (MS) in Petri dishes (100 mm diameter, 15 mm deep) in darkness at 24  $\pm$  1° C. The seedlings grew about 3–5 cm long within a week of sowing the seeds. The position of the shoot tip of the seedling inside the coleoptile could be determined by the localized enlargement of the seedling at the junction of mesocotyl and coleoptile. Sections about 5 mm long of seedlings containing a shoot tip, three to five leaf primordia, and a portion of young leaf and stem immediately below the leaf primordia were excised and cultured on MS basal medium supplemented with 500 mg/L enzymatic

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**Abbreviations:** BA = N<sup>6</sup>-benzyladenine; CH = casein hydrolysate; CSM = corn-shoot multiplication medium; 2,4-D = 2,4-dichlorophenoxyacetic acid; IBA = indole-3-butyric acid; MS = Murashige and Skoog (basal medium); MSC = multiple shoot clump(s)



**Fig. 1.** Morphogenic pathways in corn shoot-tip cultures incubated on MS medium containing various concentrations of growth regulators

casein hydrolysate (CH) and various concentrations of N<sup>6</sup>-benzyladenine (BA; 2.25, 4.5, 9, or 18 μM). The pH of the media was adjusted to 5.8 with 0.1 N sodium hydroxide, and the media were solidified with 7 g/L Phytagar (Gibco Labs, Grand Island, N.Y., USA). Three shoot tips were cultured in each Petri dish on 20 ml of medium. The explants were laid horizontally and partly buried in the medium. Four weeks later, the multiple shoot clumps (MSC) arising from these explants were divided and subcultured on MS basal medium containing 500 mg/L CH and 9 μM BA. Subsequent subcultures were also done at four-week intervals. All cultures were incubated at 25–27° C in darkness.

Shoot-multiplication cultures were also established from immature embryos of the sweet-corn cultivar, Honey N Pearl. Corn plants were first grown in a greenhouse at 27° C and open-pollinated ears were collected about 10–15 d after pollination. These immature ears were surface-sterilized with 0.5% sodium hypochlorite for 10 min and rinsed three times with sterile distilled water. Immature embryos (1–3 mm long) were extracted from the kernels and cultured on MS basal medium supplemented with CH and the same concentrations of BA as used for shoot-tip cultures.

To induce adventitious shoot formation and/or somatic embryogenesis, shoot-tip explants from one-week-old, in-vitro-germinated seedlings or shoot-tip cultures from four-week-old MSC developed on MS basal medium containing 500 mg/L CH and 9 μM BA, were transferred to MS basal medium containing 500 mg/L CH, 2.25 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.5, 9, or 18 μM BA and agar. Each of these cultures was subcultured once on the same medium after four weeks.

To test the regenerative response of different corn genotypes, 50 shoot-tip explants from seedlings of each of the genotypes listed

in Table 1 were cultured on MS basal medium supplemented with various concentrations of BA (2.25, 4.5, 9, 18 μM) with or without 2,4-D (2.25 μM). The cultures were visually scored at the end of eight weeks to estimate the relative efficiency of multiple shoot formation.

Samples of shoot and somatic embryogenic cultures were fixed in 3% glutaraldehyde, dehydrated in an ethanol series, critical-point dried, and coated with gold. Scanning electron micrographs were obtained with a JEOL (Tokyo, Japan) JSM 31 scanning electron microscope (Klomprens et al. 1986).

Green corn shoots were obtained from the dark-grown MSC and from embryogenic cultures after transfer to MS basal medium containing 500 mg/L CH, 2.25 μM BA, and 1.8 μM indole-3-butyric acid (IBA) and exposure to continuous light (60 μmol (quanta)·m<sup>-2</sup>·s<sup>-1</sup> from cool-white 40W Econ-o-watt fluorescent lamp; Philips Westinghouse, USA). Corn plantlets were rooted in Magenta GA 7 vessels (65 mm·65 mm·100 mm; Magenta Corp., Chicago, Ill., USA) containing 50 ml MS basal medium supplemented with 3.6 μM IBA and agar. Mature corn plants were obtained after transplanting these rooted plantlets into a soil mixture composed of 1:1 (v/v) peat and perlite and fertilizing weekly with Peters 20:20:20 fertilizer (W.R. Grace & Co., Cambridge, Mass., USA).

All chemicals used in this research including the MS basal medium were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

## Results

The morphogenic pathways of corn-seedling shoot tips cultured on MS media containing BA and 2,4-D are summarized in a flow diagram (Fig. 1).

**Shoot-multiplication cultures.** Preliminary experiments showed that shoot tips excised from seedlings of any age, as well as tips from the terminal and axillary shoots of greenhouse-grown corn plants, all produced MSC in the shoot-multiplication medium (data not shown). For the experiments reported here sections of one-week-old corn seedlings containing shoot tips were used as explants primarily because the position of the shoot tip

**Fig. 2.** Formation of multiple shoots from a shoot-tip explant of corn two weeks after culture on CSM. × 11

**Fig. 3.** An MSC four weeks after culture on CSM. × 20

**Fig. 4.** Scanning electron micrograph of a section of MSC four weeks after incubation in darkness on CSM. *L*, leaf; *LH*, leaf hair; *ST*, shoot tip. × 20; bar = 500 μm

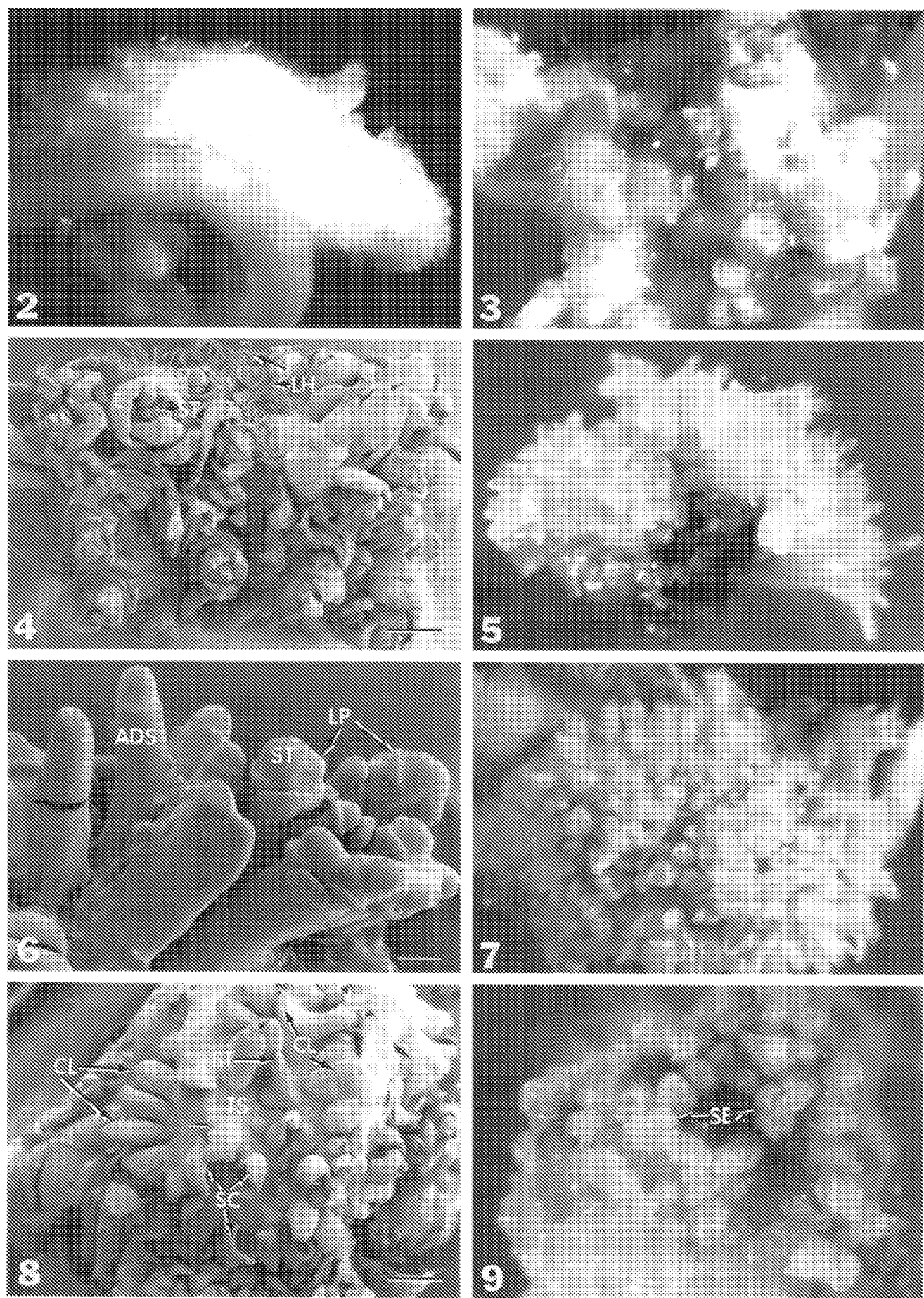
**Fig. 5.** Adventitious shoot formation from a section of MSC after eight weeks of culture on CSM supplemented with 2.25 μM 2,4-D. × 16

**Fig. 6.** Scanning electron micrograph of a section of MSC shown in Fig. 5. *ADS*, adventitious shoot; *LP*, leaf primordium; *ST*, shoot tip. × 40; bar = 200 μm

**Fig. 7.** Direct somatic embryogenesis from a shoot-tip culture eight weeks after incubation on MS medium containing 500 mg/L CH, 2.25 μM 2,4-D and 4.5 μM BA. × 16

**Fig. 8.** Scanning electron micrograph of a culture showing the formation of scutella and coleoptiles from the tissue stratum. *CL*, coleoptile; *SC*, scutellum; *ST*, shoot tip; *TS*, tissue stratum. × 20; bar = 500 μm

**Fig. 9.** Indirect somatic embryogenesis in callus cultures derived from an MSC. *SE*, somatic embryo. × 16



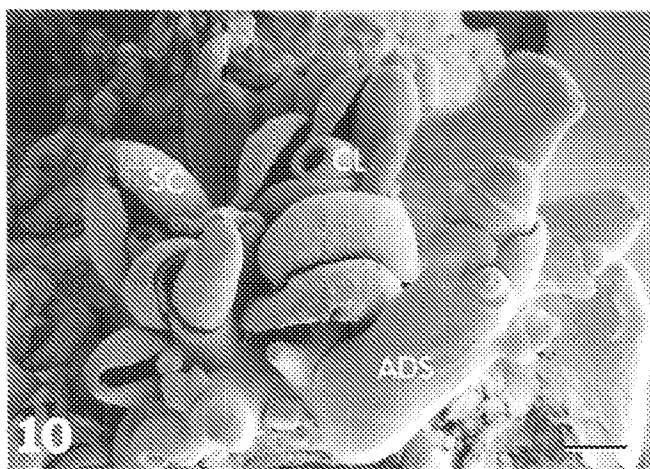
in the seedlings can easily be recognized by a localized visible enlargement.

Shoot tips began to produce MSC within one to two weeks of culture on MS basal medium containing 500 mg/L CH and various concentrations of BA. During the initial culture, shoot multiplication from shoot tips occurred inside the leaves, and therefore, the shoot clumps were not visible until the leaf sheath of the explants was removed (Fig. 2). Shoot multiplication was observed from shoot-tip explants on medium with each of the BA concentrations tested (2.25–18  $\mu$ M), but the maximum number of normal shoots was obtained at 9  $\mu$ M BA. Therefore, MS basal medium supplemented with 500 mg/L CH and 9  $\mu$ M BA was designated as corn-shoot-multiplication medium (CSM) (Table 1). It appears that rapid activation of axillary buds with little internode elongation followed by differentiation of adventitious shoots from the shoot meristem without apparent callus formation produced the MSC in each shoot-tip culture (Fig. 3). Shoots multiplied as tight clusters of 0.5–2-mm-long shoots each with two or three visible leaf primordia, and the number of shoots per MSC could be counted only under a binocular stereomicroscope. About 20–50 shoots were produced per

shoot-tip explant within four weeks of culture (Fig. 4). Each of these shoot clumps was divided into ten units, each unit containing two to five tiny shoots. These shoot-clump units were subcultured on fresh CSM every four weeks. At the end of the first subculture (about nine weeks after sowing the seeds), each seedling shoot tip had produced about 500 shoot apices.

Shoot-multiplication cultures have been maintained for a year by regular division and subculture of shoot clumps onto fresh CSM every four weeks. Repeated subculture of shoot cultures onto CSM produced more compact shoot clusters because of the very high frequency of shoot initiation. Scanning electron micrographs showed the crowded shoot clumps and the formation of trichomes (hairs) on the leaves (Fig. 4). Those shoot primordia which were trapped in the middle of the crowded-shoot clumps tended to produce abnormal shoots with twisted leaves. However, these apparently abnormal shoots eventually grew into normal plants when they were transferred onto MS medium lacking BA and were exposed to light (not shown).

Multiple shoot clumps were also produced from the precociously-germinated-seedling shoot apices of in-vitro-cultured immature zygotic embryos of Honey N



**Fig. 10.** Scanning electron micrograph of a mixture of adventitious shoots and somatic embryos in an embryogenic culture of corn shoot tip. ADS, adventitious shoot; CL, coleoptile; SC, scutellum.  $\times 20$ ; bar = 200  $\mu$ m



**Fig. 11.** Shoot development from MSC after transfer to MS medium containing 2.25  $\mu$ M BA and 1.8  $\mu$ M IBA.  $\times 0.6$

**Fig. 12.** A fertile corn plant regenerated from an MSC.  $\times 0.08$

Pearl when cultured on CSM. These shoot cultures also displayed a similar intensity of shoot multiplication to that described for shoot-tip cultures of seedlings.

When the shoot-tip explants from in-vitro-germinated seedlings and shoot-clump units from the MSC of Honey N Pearl were transferred to CSM supplemented with 2.25  $\mu$ M 2,4-D and incubated in darkness, the shoot tip enlarged and differentiated shoot clumps containing many adventitious shoots (Figs. 5, 6). The number of adventitious shoots that were produced per shoot-tip explant was relatively high when the culture was on medium containing a combination of 2,4-D and BA as compared to shoot tips cultured on BA alone. Moreover, adventitious shoots that were produced in the presence of 2,4-D and BA remained smaller, and leaf elongation was minimal. There was no apparent callus formation preceding the adventitious-shoot multiplication.

*Direct somatic embryogenesis from shoot-tip cultures.* Compact and organized tissue layers with a smooth epidermis were produced from each shoot tip cultured either in light or in darkness on MS medium containing 500 mg/L CH, 2.25  $\mu$ M 2,4-D and 4.5  $\mu$ M BA (hereafter

called embryogenic medium) within about eight weeks of incubation. These organized-tissue layers, which will be hereafter called "tissue stratum" as suggested by Wernicke et al. (1982), differentiated into a virtual lawn of embryos (Fig. 7). The scutella of the embryos initiated first, and the preservation of cellular integrity of the epidermis of the tissue layer during the initiation of scutella was observed under the scanning electron microscope. Somatic embryos emerged directly from the tissue stratum without apparent callus formation (Fig. 8). The leaf-like scutella and the tubular coleoptiles differentiated at random from the sub-epidermal cells of the tissue stratum by expanding and folding without disrupting the outer layers of the tissue stratum (Fig. 8). Therefore, the scutella were spatially separated from the coleoptiles. A row of coleoptiles could be seen without the subtending scutella in the scanning electron microscope. Most of the coleoptiles seemed to be long and hollow structures, while a few initiated visible shoot tips (Fig. 8). The root poles of the so-called embryos were not apparent and may have been merged with the tissue stratum. Shoot tips cultured on MS medium containing 500 mg/L CH and 2.25  $\mu$ M 2,4-D alone produced only non-morphogenic calli.

**Table 1.** Relative efficiency of shoot multiplication in seedling shoot-tip cultures of corn genotypes. The cultures were evaluated following eight weeks of continuous culture on agar-solidified MS basal medium supplemented with 500 mg/L CH and different concentrations of BA with or without 2,4-D and incubated at 25–27° C in darkness.  
Number of shoots per shoot tip culture  
– = <10, + = 10–20, ++ = 20–50,  
+++ = > 50

2,4-D BA	Growth regulator concentration ( $\mu$ M)						
	0 2.25	0 4.5	0 9.0	0 18.0	2.25 4.5	2.25 9.0	2.25 18.0
<i>Sweet corn genotypes<sup>a</sup></i>							
EXP 4427	–	+	++	–	+++	+++	+++
Honey N Pearl	+	++	+++	+	++	+++	+++
Illinois Gold Xtra Sweet	+	++	++	++	+++	+++	+++
<i>Michigan genotypes<sup>b</sup></i>							
509	–	+	++	+	++	++	++
420	–	+	+	–	–	++	–
5922	–	+	+	–	–	++	+++
582	–	+	+	+	++	++	++
579	–	–	+	–	++	++	++
482	+	++	++	++	+++	+++	+++
466	–	+	+	++	++	++	++
<i>Illinois genotypes<sup>a</sup></i>							
B73	–	+	+	+	+++	+++	+
M79	+	+	+	–	++	++	++
VA22	–	+	+	–	+++	++	++
CM 105	–	+	+	–	–	++	–
B84	–	–	+	–	+	+	–
PA 91	–	+	+	+	++	–	–
FR 634	–	+	+	–	++	+	–
FR 632	–	–	+	–	+	++	++
FRM 017	+	+	+	+	++	++	–
<i>Minnesota genotype<sup>c</sup></i>							
A188	–	+	+	–	–	–	++

Seeds were obtained from

<sup>a</sup> Illinois Foundation Seeds, Champaign, Ill., USA.

<sup>b</sup> Michigan Agricultural Experiment Station, East Lansing, USA.

<sup>c</sup> Crop Breeding Project, Department of Agronomy, University of Minnesota, St. Paul, USA.

**Indirect somatic embryogenesis from callus cultures.** When pieces of the adventitious shoot cultures and direct-embryo-forming cultures were transferred to embryogenic medium, friable calli were produced followed by somatic embryogenesis when examined after eight weeks culture. These somatic embryos turned green after one week of exposure to light (Fig. 9). Mixtures of both adventitious shoots and somatic embryos were observed in some cultures (Fig. 10).

**Response of different genotypes.** All 20 corn genotypes tested produced multiple-shoot cultures on CSM (9  $\mu$ M BA) (Table 1). Shoot tips from 18 out of 20 genotypes tested produced at least 10 shoots per tip when cultured on CSM supplemented with 2.25  $\mu$ M 2,4-D. More than half of the genotypes did not respond to the lowest (2.25  $\mu$ M) and the highest (18  $\mu$ M) concentrations of BA. Preliminary experiments showed that 2,4-D alone failed to induce multiple-shoot formation from shoot-tip explants. In general, shoot regeneration in most genotypes was high on media containing both 2,4-D and BA. The shoots formed on media containing combinations of 2,4-D and BA may have been derived either from somatic embryos, from adventitious buds, or from both.

**Shoot elongation and rooting.** Regardless of the exact morphogenic pathway, normal shoots were formed when the shoot-multiplication cultures and embryogenic cultures were exposed to light. However, for optimum shoot elongation, shoot clusters and somatic embryos had to be transferred to MS basal medium containing 2.25  $\mu$ M BA and 1.8  $\mu$ M IBA (Fig. 11). When the shoots were 4–6 cm long with three or four leaves, they were rooted on MS basal medium containing 3.6  $\mu$ M IBA. Shoot elongation and rooting did not require CH in the medium. About 80% of the shoots or somatic embryos developed into fertile plants (Fig. 12). The other regenerants from MSC and from the somatic embryogenic cultures did not develop into plants possibly because of the nutritional limitations imposed by the crowded shoots or embryos.

## Discussion

Plant regeneration through somatic embryogenesis has previously been achieved in *Zea* mostly from in-vitro cultures of 1–2-mm-long-immature zygotic embryos excised precisely 10–15 d after fertilization (Green and Phillips 1975; Springer et al. 1979; Duncan et al. 1985; Vasil et al. 1985; Hodges et al. 1986). Cultures of tassel primordia (Rhodes et al. 1986), mature seed embryos (Wang 1987), and leaf segments (Conger et al. 1987) of corn have also regenerated plants through somatic embryogenesis. Our work shows that the corn-shoot meristem can be committed to form either clumps of multiple shoots or somatic embryos in vitro by manipulating the concentration of BA and 2,4-D in the culture medium. The demonstration that corn-shoot meristems are committed to form either shoots or somatic embryos (Figs. 2–10) supports the concept of McDaniel and Poethig (1988) that organ formation from apical mer-

istem cells in corn is determined just before the organ begins to be initiated. Sorghum shoot tips also similarly differentiated somatic embryos in the presence of 11  $\mu$ M 2,4-D and 2.25  $\mu$ M kinetin, another cytokinin, while cultured on 45  $\mu$ M kinetin alone they produced only shoots (Bhaskaran and Smith 1990). Cytokinins, particularly benzyladenine, are widely used to obtain proliferation of multiple shoots from shoot-tip cultures of many plants (see review by Hu and Wang 1983).

Somatic embryos of corn arise either directly from the scutellar tissue of the cultured immature zygotic embryos (Vasil et al. 1985) or indirectly from embryogenic calli derived from scutella (Springer et al. 1979). Both pathways of somatic embryogenesis do occur in corn shoot-tip cultures (Figs. 7–9), but the growth regulators which mediate this process vary, 2,4-D triggering somatic embryogenesis in cultures of immature zygotic embryos but both BA and 2,4-D being needed for embryogenesis in shoot-tip cultures. Exogenous cytokinins improve somatic embryogenesis in plants (see review by Raghavan 1986). Somatic embryos were regenerated in clover cultures (Maheswaran and Williams 1985) using BA as sole growth regulator, while both BA and 2,4-D or naphthalene-1-acetic acid are required for rye grass (Dale 1980), tobacco (Stolarz et al. 1991) and corn shoot-tip cultures (Table 1).

De-novo differentiation of shoot apices observed in our shoot-tip cultures as well as on the surface of the scutellar tissue of cultured immature zygotic embryos of corn by Springer et al. (1979) was actually the result of somatic embryogenesis (Vasil et al. 1985). In sorghum (Wernicke et al. 1982) and in wheat (Wernicke and Milkovits 1986), both the scutella and the shoot apical meristems produce a “tissue stratum” (compact, organized tissue layer with a smooth epidermis) which, by folding and budding, produces somatic embryos without apparent callusing. The somatic embryos produced in our corn shoot-tip cultures appear also to have developed in such a manner (Figs. 7, 8). Anomalous embryogenesis does occur in corn and in other cereal cell cultures (Wernicke and Milkovits 1986). Occurrence of both bipolar (somatic embryos) and unipolar (adventitious shoots) structures in close proximity is a common occurrence in cell cultures of corn, rice and sorghum (Vasil 1987; Jones and Rost 1989; Bhaskaran and Smith 1990). The scutellum is a modified leaf (Esau 1977, chpts. 2, 15, 16) and the leafy nature of the scutellum in the precociously-germinating somatic embryos can be seen in the scanning electron micrographs of the corn cultures (Figs. 7, 8). Spatial separation of scutella and embryonic axes (Fig. 8) and contiguous formation of multiple coleoptiles (Fig. 7) with or without subtending scutella were frequently observed in embryogenic cultures of other grasses (Vasil 1987). Somatic embryos do not develop in a controlled ovular environment like zygotic embryos. Therefore complete organization of somatic embryos is seldom observed in many grasses (He et al. 1990).

Genotypic differences in plant regeneration in corn cultures can be circumvented by altering the concentration of growth regulators (Vasil 1987) in the culture medium. Likewise shoot tips of corn cultured on 2,4-D

alone failed to regenerate plants, but BA alone or in combination with 2,4-D induced regeneration of plants in all 20 corn genotypes tested. The requirement of BA both for shoot formation and for somatic embryogenesis in several corn genotypes indicates that cytokinin may be a key regulator of morphogenesis in shoot-tip cultures of corn.

Heng Zhong is a Rockefeller Foundation Fellow on leave from the Institute of Botany, Academia Sinica, Beijing, P.R. China. This work was supported by a grant from the Midwest Plant Biotechnology Consortium and U.S.-A.I.D. grant No. DAN-4197-A-00-1126-00 to M.B. Sticklen. Thanks are due to Illinois Foundation Seeds, Minnesota Crop Improvement Association, and Michigan Agricultural Experiment Station, for the gift of corn seeds used in this research. Critical review of this manuscript by Indra K. Vasil, University of Florida, Gainesville; Wesley P. Hackett, University of Minnesota, St. Paul; Scott Poethig, University of Pennsylvania, Philadelphia; and Mark G. Bolyard, Michigan State University is gratefully acknowledged. The authors would also like to thank the Services of Center for Electron Optics, Michigan State University, for the electromicroscopic work as related to this publication.

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## **EXHIBIT B**



US005736369A

**United States Patent** [19][11] **Patent Number:** **5,736,369****Bowen et al.**[45] **Date of Patent:** **Apr. 7, 1998****[54] METHOD FOR PRODUCING TRANSGENIC CEREAL PLANTS**

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[21] Appl. No.: **483,091**

[22] Filed: **Jun. 7, 1995**

**Related U.S. Application Data**

[63] Continuation of Ser. No. 282,270, Jul. 29, 1994, abandoned.

[51] Int. Cl.<sup>6</sup> ..... C12N 15/00; C12N 15/05; A01H 4/00

[52] U.S. Cl. .... **435/172.3**; 435/172.1; 435/412; 435/424; 435/430; 47/58; 47/DIG. 1; 800/205; 800/DIG. 56

[58] Field of Search ..... 800/200, 205, 800/250, DIG. 56; 47/58; 435/172.3, 172.1, 240.4, 145, 149, 412, 424, 430

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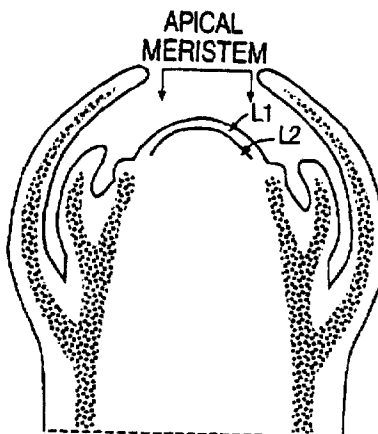
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**[57] ABSTRACT**

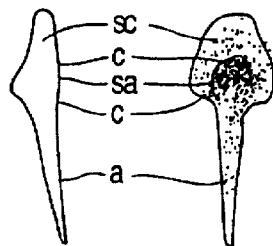
To obtain a transgenic cereal plant which is stably transformed, an exposed cereal meristem is subjected to biolistic bombardment in order to target non-differentiated meristem cells for transformation. Immature embryos at the early proembryo, mid proembryo, late proembryo, transitional or early coleoptilar stage are harvested for biolistic bombardment. The meristem tissue or cells fated to contribute to the meristem then are manipulated in order to enlarge transgenic sectors, either through selection and/or through effecting a proliferation from the tissue of shoots or multiple meristems per se. The shoot population thus obtained then is screened, by means of a nonlethal enrichment assay, to identify either chimeric sectors that will contribute to germline transmission, or non-sectored, L2 periclinal chimeras that will by definition transmit to progeny. Increased time in culture, under selection, enhances the prospects for sectoral-to-periclinal conversions, and also selects for L1-to-L2 conversions which, through a shift in position, ultimately contribute to the germline. Transgenic sectors also are stabilized during the step of tillering.

**17 Claims, 2 Drawing Sheets**

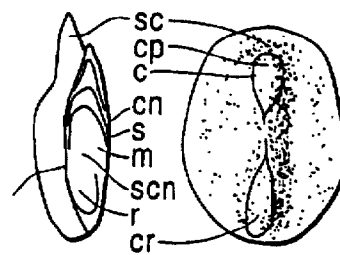
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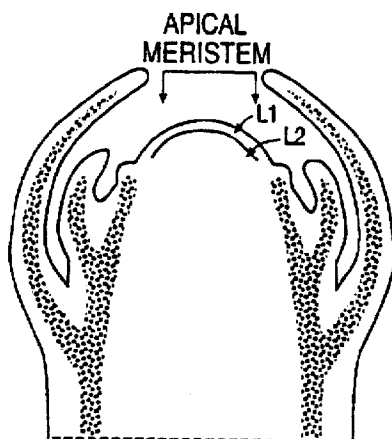
**FIG. 1a**



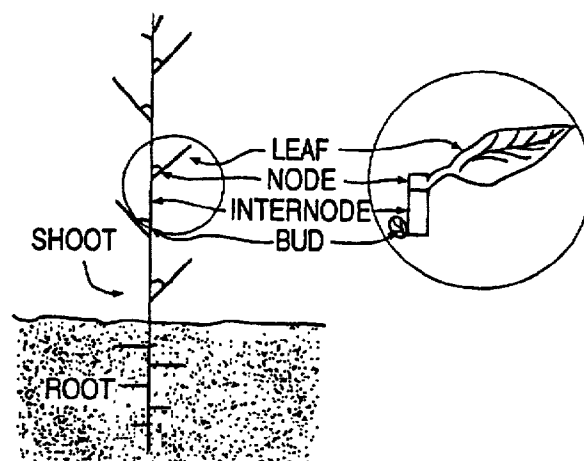
**FIG. 1b**



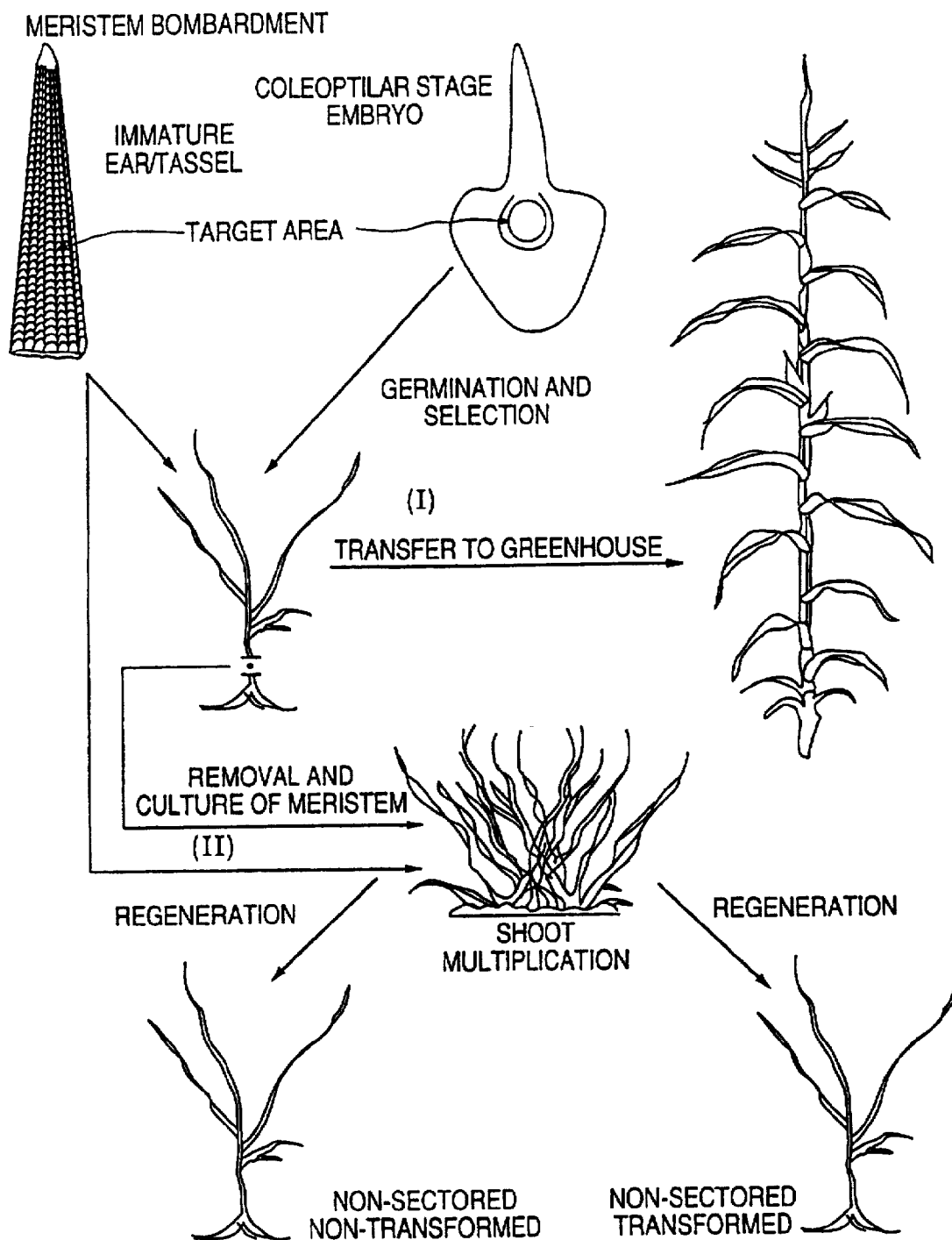
**FIG. 1c**



**FIG. 1d**



**FIG. 2**



## METHOD FOR PRODUCING TRANSGENIC CEREAL PLANTS

This application is a continuing application that claims benefit under 35 USC §120 to application Ser. No. 08/282,270, filed Jul. 29, 1994 now abandoned, the contents of which are hereby incorporated by reference.

### BACKGROUND OF THE INVENTION

The present invention relates to obtaining plants by a methodology that entails the biolistic bombardment of meristem tissue, at a very early stage of development, and the selective enhancement of transgenic sectors, toward genetic homogeneity, in cell layers that contribute to germline transmission.

Production of transgenic plants first became routine through the use of *Agrobacterium*, and the use of this vector with totipotent tissues has become the method of choice for many dicotyledonous species. While steady progress has been made in expanding the genotype and species range of this method, *Agrobacterium*-mediated transformation has not been widely utilized for monocotyledonous species, including cereals, and is likely in the near term to remain restricted to specific genotypes. Similarly, protoplast-based methods are not widely applicable for monocots.

The first reports which appeared on biolistics-mediated production of fertile, transgenic maize were restricted to a specific hybrid, A188 x B73. See Gordon-Kamm et al., *Plant Cell* 2: 603 (1990), and Fromm et al., *Bio/Technology* 8: 833 (1990). Since then the technique has been extended to many important monocot crops, including barley, wheat, rice and oats, and the useful range in maize has been expanded slowly to include a handful of genotypes, for example, the commonly used A188 x B73, H99, FR16 and Pa91 genotypes. This work generally has revolved around a common theme, which is the initiation of regenerable callus from the scutellum of the embryo. In particular, all the reports in this context have highlighted a prerequisite of initiating regenerable callus from the scutellum of the immature embryo, regardless of whether there is bombardment (i) of the scutellum just after embryo isolation, followed by selection of the callus grown from the scutellar cells, (ii) of freshly initiated callus after a short preculture of the scutellum or (iii) of long-term callus or cell suspension cultures.

Progress in expanding the callus-based approaches to new genotypes or species has occurred via adaptations of the basic method to accommodate differences in morphology and growth patterns that typify different forms of immature, embryo-derived callus, i.e., friable callus versus compact callus, also referred to as Type II and Type I, respectively. Genotype restrictions remain, however, because some germplasm does not produce an appropriate callus response.

With the advent of biolistics-mediated transformation, numerous groups have explored the possibility of using microprojectile-delivery methods with meristem tissues. It has remained "an open question," however, as to "whether integrative transformation in cells of the shoot apical meristem of [a] monocotyledonous species is [even] possible." Bilang et al., *Plant J.* 4: 735 (1993).

The literature is marked by speculation concerning barriers to transforming meristem target cells which may explain the lack of success in this area. It has been observed, for example, that cereal "shoot meristems are tiny (about 100 µm) and . . . biolistic particles hit large target areas at random," and that "meristematic cells may [have] molecular mechanisms which prevents [sic] integration of foreign

DNA . . ." Potrykus, *Nature* 355: 568, 569 (1992). More generally, the fact that monocot plant species tend to display less developmental plasticity than dicot species has engendered an expectation that monocots are less amenable to stable transformation by biolistic and other techniques.

Given the lack of developmental plasticity in cereals, therefore, the historical focus of transformation efforts in these crops has been on callus derived from one of the few genotypes that produce Type I or Type II embryogenic callus. These transformation targets were subject to easy use because a large population of undetermined, proembryogenic cells could be selected. Accordingly, many research groups have taken advantage of this approach and have not pursued alternative target tissues. In particular, no one to date has reported germline transformation via meristem bombardment of maize, a key cereal crop. Lack of success in this regard has been ascribed to the rigid developmental fate of the cells composing the meristem.

### SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a methodology for the reproducible production of stably transformed cereal plants.

In accomplishing this object and others, there has been provided, in accordance with one aspect of the present invention, a method for producing transgenic cereal plants, e.g., maize, sorghum, wheat, barley, oat or rice plants, that will transmit introduced DNA to progeny, comprising the steps of

(A) introducing foreign DNA into cells selected from the group consisting of (i) cells of a meristem that is not enclosed by sheathing leaves and (ii) cells fated to contribute to said meristem; then

(B) inducing reorganization of said meristem to increase transgenic sector size, whereby the likelihood that a transgenic sector will contribute to germline transmission is increased; and thereafter

(C) exposing said meristem to conditions under which it differentiates to form a plantlet, wherein said plantlet contains said transgenic sector or is homogeneously transformed by said foreign DNA, such that said plantlet can be grown into a transformed cereal plant that will transmit said foreign DNA to progeny.

The foreign DNA can be introduced into a plurality of meristems, at least some of which differentiate in step (C) to form a plurality of plantlets. The foreign DNA is introduced into a meristem that is not enclosed by sheathing leaves including meristems from early proembryo, mid proembryo, late proembryo, transitional and early coleoptilar stage embryos.

In one preferred embodiment, reorganization is effected through at least one manipulation selected from the group consisting of (i) imposition of a nonlethal selective pressure on the meristems, (ii) mechanically-induced meristem reorganization, and (iii) hormonally-induced shoot multiplication. In another preferred embodiment the conditions in step (C) are such that the meristems undergo maturation and plant differentiation to form shoot apices, and the method further comprises effecting reorganization of meristem tissue in the shoot apices to enlarge transformed sectors or to produce periclinal L2 chimeras. The reorganization in this regard can be effected, for example, by exposing the shoot apices to nonlethal selection pressure such that transformed cells have a competitive growth advantage over nontransformed cells in the shoot apices, and the proportion of transformed cells in the shoot apices is increased. In yet

another preferred embodiment, the method further comprises a step before step (B), e.g., before step (A), of wounding the apical dome selectively. A method of the present invention also can comprise the further steps of (i) dissecting out an axillary bud from above the base of a leaf of a plantlet when a chimeric sector is observed in a substantial portion of the leaf, and then (ii) germinating the axillary bud into a whole plant or subjecting the axillary bud to shoot multiplication.

In yet another preferred embodiment, the transgenic sector of a plantlet is stabilized by inducing tillers. The apex of a transgenic plantlet is removed, the wounded plantlet is grown to induce formation of a plurality of tillers, and transgenic tillers then are selected from that plurality.

In accordance with another aspect of the present invention, a transgenic cereal plant is provided that (A) is the product of a method as described above, (B) transmits introduced DNA to progeny and (C) belongs to a cereal line that is recalcitrant to callus-based transformation. In a preferred embodiment, the transgenic cereal is a maize plant that is not produced by transformation of a genotype selected from the group consisting of A188, A188 x B73, H99, Pa91, FR16 and a genotype obtained from a cross involving any of the foregoing.

According to still another aspect of the present invention, a maize plant is provided that transmits introduced DNA to progeny and that has a pedigree including a line selected from the group consisting of PHT47, PHP02, PHV78, PHK05, PHW20, PHR62, PHN37, PHM10, PHV37, PHJ65, PHBW8, PHK29, PHJ33, PHP60, PHN73 and PHHV4.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. Indeed, various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1a-d is a series of line drawings depicting the structure of a typical cereal embryo (a) at the coleoptilar stage, which in maize occurs approximately 8 to 14 days after pollination, and (b) at the later third stage (about 22 to 28 days after pollination in maize), respectively; (c) the model shoot tip of an angiosperm, including cereals, shown in longitudinal section; and (d) the shoot and root structures which pertain in cereals generally, with a unit phytomer of the shoot highlighted. Abbreviations: c=coleoptile; cn=coleoptilar node; cp=coleoptilar pore; cr=coleorhiza; m=mesocotyl; r=primary root primordium; s=suspensor; sa=shoot apex; sc=scutellum; sen=scutellar node; sr=seminal root primordium.

FIG. 2 is a schematic representation of transformation methodology according to the present invention.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been discovered that the difficulties discerned previously in relation to applying a meristem-based transformation strategy to cereals can be overcome by (A) biolistically targeting cells of the shoot apical meristem under conditions such that the apical dome of the apical meristem is not enclosed by sheathing leaves, as depicted in FIG. 1; and (B) using a nonlethal selection regimen to effect

an enrichment of transformed cells, for example, by giving transformed cells a competitive advantage over nontransformed cells, and thereby to facilitate an increase in sector width. The nonlethal selection also can promote the development of a short-lived, mericlinal L2 event into a stable, periclinal event in which most or all of the cells contributing to the germ line (i.e., the L2 layer) are transformed. In addition, it has been found that selective pressure can promote L1- to -L2 conversion events, thus increasing the probability of germ line transmission.

The shoot apical meristem in cereals is highly variable between species. In most species, however, a stratified meristem exists that is composed of two or three visible layers which generate the entire shoot: a superficial L1, a subsurface L2 and, in some cases, a deeper L3 layer. The outer layer(s) comprise the tunica, which are characterized by anticlinal cell divisions. In contrast, divisions in the innermost layer, the corpus, occur randomly, both anticlinally and periclinally. In maize the meristem is believed to be composed of only two layers, L1 and L2, and possibly a third L3 layer. See Poethig, CONTEMPORARY PROBLEMS IN PLANT ANATOMY 235-39 (1984). Cell differentiation to delimit the major tissues of the shoot is position-dependent rather than lineage-dependent.

For example, in most species the epidermis is generated almost exclusively by the L1 layer, with the L2 layer contributing to the germ line. In the process of introducing a foreign gene into a subset of apical meristem cells, one creates a plant that necessarily is "chimeric," i.e., a plant in which portions have been altered in genetic composition. There are three major categories of chimeric plants, based on the characteristic pattern of genetic differences: (1) sectoral chimeras, in which a portion of the plant is "genetically distinct" through all cell layers by virtue, for example, of displaying a mutant somatic phenotype, a change in chromosome number, or the presence of transformed cells; (2) periclinal chimeras, in which an entire cell layer (L1 alone or L2 alone, for instance) is different from the rest of the plant; and (3) mericlinal chimeras, which represent an intermediate between the other two types, i.e., a genetic difference characterizes only a portion of one layer.

In this description, the terms "biolistic" and "biolistically" denote an approach to genetic transformation described, for example, in U.S. Pat. Nos. 4,945,050 and 5,141,131, the respective contents of which are hereby incorporated by reference.

Pursuant to a biolistic approach, force is transmitted to small particles that carry DNA, for example, coated on particulate surfaces or absorbed into the particles, in such a way that the exerted pressure forces particles into a targeted cell or tissue ("biological sample"); the particles thus are called "microprojectiles" or "microcarriers." In other words, the microprojectiles are propelled at the biological sample, accelerating to such speed that, upon impact, they penetrate cellular surfaces and are incorporated into the interior of a cell or cells in the sample.

The microprojectiles should have an average diameter sufficiently small to permit penetration of and retention by cells of the biological sample without killing the cells. Particles of gold or tungsten, in the size range of about 0.1 to 4 microns, are illustrative of microprojectiles that are suitable for delivering exogenous nucleic acid into a host. Other types of biolistic delivery vehicles are disclosed, for example, by U.S. Pat. Nos. 5,120,657 (electrical discharge propels a carrier sheet toward target) and 5,240,842 (nucleic acid delivered via aerosol droplets), and in PCT application WO 92/01802 (ice particles as carrier).

In relation to aspect (A) mentioned above, the present invention contemplates the biolistic targeting of apical meristem cells at an early developmental stage. In a preferred embodiment, meristem cells are bombarded at a developmental phase that is no later than the coleoptile-ring stage, when the apical dome is fully exposed, lacking protection from leaf primordia, and is composed of fewer cells in the meristem than are present at later stages. The stages of maize embryogenesis are described in detail by Poethig et al., *Developmental Biology* 117: 392-404 (1986), the contents of which are incorporated by reference.

More specifically, the transformation method of the present invention focuses on coleoptilar and earlier stage embryos, namely, early proembryo, mid proembryo, late proembryo, and the transitional stages of embryo development. At the earliest stages of development, the meristem is not defined; instead, a group of cytoplasmically dense cells undergo more rapid division and, ultimately, form the apical meristem.

In targeting these various embryo stages, therefore, DNA is introduced (i) into cells that make up the meristem proper (i.e., at the coleoptilar stage) or, (ii) in the earlier stages of development, into cells that are destined, by position or fate, to contribute to the meristem. Biolistic bombardment according to the present invention is effected by orienting the embryo so that cells that are within a meristem or that are destined to contribute to the meristem are exposed directly to the biolistic projectiles.

In late proembryos, the axis side of the embryo is slightly flattened, allowing this side of the embryo to be placed face up (away from the agar) for bombardment. Transition stage and coleoptilar stage embryos are similarly oriented. There is no such orientation, however, for mid and early proembryos on agar after isolation (i.e., before shooting). Rather, when proembryos are placed in a random orientation on the agar medium, the meristem apparently develops on the upper side of the embryo (away from the medium). Thus, placement on the medium may be stimulating the embryo to re-orient its growth axis, for example, by virtue of the in vitro conditions which are provided (i.e., the new hormonal gradient that is being established within the embryo).

A convenient and, hence, preferred source of meristems for use in the present invention are coleoptilar stage embryos. At the coleoptilar stage of cereal embryo development, the coleoptile is visible as a ring of leaf primordium surrounding an exposed meristem. In maize, the early coleoptilar stage can generally be obtained 10 to 12 days after pollination. (The days-after-pollination criterion, or "DAP," is affected by embryonic environment and genotype, and therefore is an adjunct to developmental staging based on morphology, which is an important criterion for timing of transformation in the present invention.) At the early coleoptilar stage the boundary of the meristem is distinct, with a visible tunica and corpus (L1 and L2 layers, respectively).

A particularly preferred source of target cells for use in the present invention are present in the early proembryo, mid proembryo, late proembryo and the transitional stages in embryo development. In maize, the early proembryo, mid proembryo, late proembryo and the transitional stages can generally be isolated 2, 4, 7-8 and 8-10 DAP, respectively (see Poethig et al. (1986), cited above). Again, the developmental stage is the important criterion. Rate of development and, hence, DAPs at which embryos are isolated vary with growth environment and genotype.

At the mid proembryo stage there is no distinction between the L1 and L2 layers. The distinction between L1

and L2 progresses until it is well-defined by the time the embryo reaches the transitional stage.

Alternatively, immature staminate inflorescences (tassels) and pistillate inflorescences (ears) can serve as sources of meristems for transformation in accordance with the present invention. "Immature" here denotes a developmental state when floral meristems still are developmentally plastic, i.e., are capable of shoot differentiation. This developmental plasticity should be exploitable, pursuant to the present invention, for transformation of many Gramineaceous species, given the recognized similarities in inflorescence development among the grasses.

A trained technician can isolate 200 to 600 mid proembryo, late proembryo, transitional or coleoptilar-stage embryos per day, the ease of isolation and number of isolated embryos increasing with embryo size. On the order of ten times as many meristem explants can be isolated from immature tassels and/or ears, and a large percentage of these can be induced to follow a vegetative pattern of development. Another important advantage associated with using floral explants as meristem sources is that many genotypes exhibit better meristem growth and shoot multiplication when the floral explants are the starting material. This advantage is pronounced, for example, with respect to an inbred maize line with a lineage that includes line PHV78. Conversely, immature embryos are the preferred explant for some genotypes, such as maize inbreds having a lineage including line PHBW8. Access to both options significantly extends the genotype range for meristem transformation pursuant to the invention.

Whatever explant or tissue is used as a source of target cells for biolistic treatment in accordance with the present invention, the bombarded cells are subjected to a first, nonlethal selection pressure in the course of generating plantlets which are grown out directly (course I in FIG. 2) or, alternatively, are subjected to meristem reorganization, induced mechanically or hormonally, in advance of a second nonlethal selection (course II in FIG. 2). The aspects of nonlethal selection and induced meristem reorganization are addressed in greater detail below.

The developmental fate of cells within the meristem normally is rigidly determined. Thus, transformation of a particular cell within the meristem typically will result in a small transgenic sector made up only of the descendants of that cell. Without further manipulation, such sectors rarely if ever overlap gametophytic tissue during normal development. But by targeting cells at earlier developmental stages, as described above, and then applying mild selective conditions in accordance with the present invention, i.e., the pressure provides a growth advantage to transformed cells but is not severe enough to impede the overall development of the meristem, then the consequent faster division rate of transformed cells results in the descendant cells comprising a greater portion of the meristem. Accordingly, the transgenic sector contributes to a larger portion of the mature plant, and there is a greater likelihood that the sector will contribute to germline transmission.

According to one preferred embodiment, a selective growth advantage is imparted to transformed cells in the form of NPTII-encoded resistance to tobramycin, kanamycin or a related compound. It is acceptable, however, to confer resistance to another "bleaching" antibiotic (by means of a streptomycin-resistance gene, for instance) or herbicide, for example, by transformation with the *crfI* gene, which imparts resistance to norflurazon. By the same token, the present invention contemplates similar non-lethal strat-

egies which entail the use of other selective agents, such as bialaphos and hygromycin, with a corresponding, resistance-imparting gene, so long as the resulting selective pressure retards the growth of non-transformed cells relative to cells in the transgenic sector.

A model experiment in this regard would involve exposing samples of isolated meristem tissue to a graded series of dilutions of the selection agent in the medium of choice, and then determining a concentration threshold below which the selective pressure favoring transformed cells is not so stringent as to be detrimental to general meristem development. While this approach typically would result in continued meristem growth during selection, the present invention also envisages establishing conditions of little or no meristem growth ("static conditions") which are punctuated by brief exposure(s) to a higher concentration of selection agent ("pulsed selection") which otherwise would adversely affect overall meristem development.

As noted above, cereal transformation according to the present invention optionally involves a reorganization of the meristem, for example, by wounding of the apical dome. While other methods of wounding also result in reorganization, a preferred method is to pierce the apical dome using a micromanipulation needle. The reorganization thus effected alters growth in the apical dome and, it has been discovered, prompts a proliferation of multiple meristems which, in turn, enhances transformation frequency and sector size. For example, mechanically-induced meristem proliferation in conjunction with selective pressure results in an increase in frequency and size of the transgenic sectors observed in subsequent leaves.

Meristem reorganization may precede biolistic treatment, followed by germination and selection leading to the production of chimerically transformed plants (course I). Alternatively, mechanical wounding can be performed after bombardment of the meristems in order to effect a proliferation of meristems. When applied in this manner on chimeric meristems, the sectors can enlarge because the reorganized meristems are derived from a smaller number of cells and, hence, the percentage of transformed cells in the meristems is increased.

Pursuant to course II (see FIG. 2), a reorganization is brought about by hormonally-induced shoot multiplication with respect to the developing shoot meristem of a plantlet selected for the presence of a transformed sector. The hormonally induced reorganization need not be exclusive of the optional, mechanically induced reorganization mentioned above, and brings about meristem proliferation via shoot multiplication.

To effect hormonally induced reorganization, the developing shoot meristem first is localized, typically in a swelling that occurs in the germinated plantlet at the junction between the mesocotyl and the epicotyl (see FIG. 2). A section of 2 to 3 mm in size which contains the meristem then can be excised at the swelling point and cultured on a shoot proliferation medium of the sort described, for example, by Lowe et al., *Plant Science* 41: 125 (1985), and by Zhong et al., *Planta* 187: 483 (1992), respectively. To this end, meristems typically are cultured on MS medium with 2 mg/l BAP (6-benzyl-aminopurine), 3% sucrose and 9 mg/l agar. More generally, a shoot multiplication medium will utilize a cytokinin, such as Kinetin, BAP, Thidiazuron or Zeatin, at a concentration between 0.5 and 10 mg/l. A low level of auxin also may be required in some genotypes. Murashige and Skoog (MS) salts are adequate but probably not optimal, in that preliminary experiments using media

with ammonium levels higher than those in MS resulted in an improved culture response. Additional additives such as the auxin transport inhibitor, TIBA, and ethylene inhibitors like silver nitrate and cefotaxime also appear to be beneficial.

By virtue of its hormonal constituency, the shoot proliferation medium forces the generation of a few to hundreds of shoots from each excised shoot meristem, thereby increasing the likelihood of obtaining a subpopulation of shoots, some of which may arise from a transformed sector. Unlike mericlinal and sectoral chimeras, which exhibit a lower probability of germline transmission, a significant and reproducible percentage of the resulting shoots are periclinal chimeras and, hence, are "stabilized" in the sense that genetic homogeneity is promoted within a cell layer, such as the L2 layer, that ultimately contributes to germline transmission.

To identify the aforementioned shoot subpopulation, the large population of induced shoots is screened to identify non-sectored, periclinal chimeras. This is accomplished via a nonlethal assay which brings about an enrichment of transformed cells through the use of selective agents (i) that bleach normally green tissue at levels that do not inhibit growth or (ii) that inhibit growth of non-transformed meristem sectors without significantly reducing viability of the meristems.

Use of an appropriate selective agent at nonlethal levels, as described, also provides the opportunity to assess visually the extent of homogeneity within a transformed meristem layer. Increased time in culture under selection, pursuant to the present invention, enhances the prospect of mericlinal-to-periclinal conversions and of sectoral-to-homogeneously transformed conversions, and also selects for L1-to-L2 conversions which, through a shift in position, ultimately contribute to the germline.

From the preceding commentary it is apparent that one aspect of the present invention relates to forcing meristem reorganization, before bombardment, after bombardment or both, by suppressing cell growth through selective wounding of the apical dome, prompting generation of multiple meristems, or by exposing excised meristems to hormonal stimuli likewise leading to multiple meristems, albeit in the form of proliferated shoots. According to yet another preferred embodiment, the axillary bud of a transformed plantlet can be dissected out, from just above a leaf base, when a chimeric sector is observed in a substantial portion of that leaf. The isolated axillary bud represents an additional meristem that can be grown into a whole plant, or taken through a brief cycle of shoot multiplication as described, thereby to obtain a more homogeneously transformed plant.

The purpose of this approach, as for the others discussed above, is to increase the frequency of germline transmission. Thus, if a transformed sector runs through more than one leaf, it should be possible to "capture" that transformation event in an axillary bud, i.e., convert a transformed mericlinal or sectoral chimera into a periclinal or homogeneously transformed shoot.

Another method of stabilizing transgenic sectors is to induce tillers in the transformed plant. In those cases in which transgenic sectors are limited to the lowermost leaves or domains of maize plants, tillering is induced to stabilize these transgenic sectors.

By means of the present invention, a wide range of cereal varieties can be transformed stably, in a genotype-independent manner, for the first time. In maize, for example, this means that elite lines which were previously

inaccessible to transformation characterized by transmission of imparted trait(s) to seed progeny now can be genetically engineered to express various phenotypes of agronomic interest. The genes implicated in this regard include but are not limited to those categorized below.

# I. Genes That Confer Resistance To Pests or Disease And That Encode:

- (A) A *Bacillus thuringiensis* protein, a derivative thereof or a synthetic polypeptide modeled thereon. See, for example, Geiser et al., *Gene* 48: 109 (1986), who disclose the cloning and nucleotide sequence of a Bt  $\delta$ -endotoxin gene. Moreover, DNA molecules encoding  $\delta$ -endotoxin genes can be purchased from American Type Culture Collection (Rockville, Md.), under ATCC accession Nos. 40098, 67136, 31995 and 31998.
- (B) A lectin. See, for example, the disclosure by Van Damme et al., *Plant Molec. Biol.* 24: 825 (1994), who disclose the nucleotide sequences of several *Clivia miniata* mannose-binding lectin genes.
- (C) A vitamin-binding protein such as avidin. See U.S. patent application Ser. No. 07/911,864, the contents of which are hereby incorporated by reference. The application teaches the use of avidin and avidin homologues as larvicides against insect pests.
- (D) An enzyme inhibitor, for example, a protease inhibitor or an amylase inhibitor. See, for example, Abe et al., *J. Biol. Chem.* 262: 16793 (1987) (nucleotide sequence of rice cysteine proteinase inhibitor), Huub et al., *Plant Molec. Biol.* 21: 985 (1993) (nucleotide sequence of cDNA encoding tobacco proteinase inhibitor I), and Sumitani et al., *Biosci. Biotech. Biochem.* 57: 1243 (1993) (nucleotide sequence of *Streptomyces nitrosporeus*  $\alpha$ -amylase inhibitor).
- (E) An insect-specific hormone or pheromone such as an ecdysteroid and juvenile hormone, a variant thereof, a mimetic based thereon, or an antagonist or agonist thereof. See, for example, the disclosure by Hammock et al., *Nature* 344: 458 (1990), of baculovirus expression of cloned juvenile hormone esterase, an inactivator of juvenile hormone.
- (F) An insect-specific peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest. For example, see the disclosures of Regan, *J. Biol. Chem.* 269: 9 (1994) (expression cloning yields DNA coding for insect diuretic hormone receptor), and Pratt et al., *Biochem. Biophys. Res. Comm.* 163: 1243 (1989) (an allostatin is identified in *Diploptera puntata*). See also U.S. Pat. No. 5,266,317 to Tomalski et al., who disclose genes encoding insect-specific, paralytic neurotoxins.
- (G) An insect-specific venom produced in nature by a snake, a wasp, etc. For example, see Pang et al., *Gene* 116: 165 (1992), for disclosure of heterologous expression in plants of a gene coding for a scorpion insectotoxic peptide.
- (H) An enzyme responsible for an hyperaccumulation of a monoterpene, a sesquiterpene, asteroid, hydroxamic acid, a phenylpropanoid derivative or another non-protein molecule with insecticidal activity.
- (I) An enzyme involved in the modification, including the post-translational modification, of a biologically active molecule; for example, a glycolytic enzyme, a proteolytic enzyme, a lipolytic enzyme, a nuclease, a cyclase, a transaminase, an esterase, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an elastase, a chitinase and a glucanase, whether natural

- or synthetic. See PCT application WO 93/02197 in the name of Scott et al., which discloses the nucleotide sequence of a callase gene. DNA molecules which contain chitinase-encoding sequences can be obtained, for example, from the ATCC under accession Nos. 39637 and 67152. See also Kramer et al., *Insect Biochem. Molec. Biol.* 23: 691 (1993), who teach the nucleotide sequence of a cDNA encoding tobacco hookworm chitinase, and Kawalleck et al., *Plant Molec. Biol.* 21: 673 (1993), who provide the nucleotide sequence of the parsley ubi4-2 polyubiquitin gene.
- (J) A molecule that stimulates signal transduction. For example, see the disclosure by Botella et al., *Plant Molec. Biol.* 24: 757 (1994), of nucleotide sequences for mung bean calmodulin cDNA clones, and Griess et al., *Plant Physiol.* 104: 1467 (1994), who provide the nucleotide sequence of a maize calmodulin cDNA clone.
  - (K) A hydrophobic moment peptide. See U.S. patent applications Ser. No. 08/168,809 (disclosure of peptide derivatives of Tachyplesin which inhibit fungal plant pathogens) and serial No. 08/179,632 (teaches synthetic antimicrobial peptides that confer disease resistance), the respective contents of which are hereby incorporated by reference.
  - (L) A membrane permease, a channel former or a channel blocker. For example, see the disclosure by Jaynes et al., *Plant Sci.* 89: 43 (1993), of heterologous expression of a cecropin- $\beta$  lytic peptide analog to render transgenic tobacco plants resistant to *Pseudomonas solanacearum*.
  - (M) A viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral infection and/or disease development effected by the virus from which the coat protein gene is derived, as well as by related viruses. See Beachy et al., *Ann. Rev. Phytopathol.* 28: 451 (1990). Coat protein-mediated resistance has been conferred upon transformed plants against alfalfa mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus. Id.
  - (N) An insect-specific antibody or an immunotoxin derived therefrom. Thus, an antibody targeted to a critical metabolic function in the insect gut would inactivate an affected enzyme, killing the insect. Cf. Taylor et al., Abstract #497, *SEVENTH INT'L SYMPOSIUM ON MOLECULAR PLANT-MICROBE INTERACTIONS* (1994) (enzymatic inactivation in transgenic tobacco via production of single-chain antibody fragments).
  - (O) A virus-specific antibody. See, for example, Tavladoraki et al., *Nature* 366: 469 (1993), who show that transgenic plants expressing recombinant antibody genes are protected from virus attack.
  - (P) A developmental-arrestive protein produced in nature by a pathogen or a parasite. Thus, fungal endo  $\alpha$ -1,4-D-polygalacturonases facilitate fungal colonization and plant nutrient release by solubilizing plant cell wall homo- $\alpha$ -1,4-D- galacturonase. See Lamb et al., *Bio/Technology* 10: 1436 (1992). The cloning and characterization of a gene which encodes a bean endopolygalacturonase-inhibiting protein is described by Toubart et al., *Plant J.* 2: 367 (1992).

(Q) A developmental-arrestive protein produced in nature by a plant. For example, Logemann et al., *Bio/Technology* 10: 305 (1992), have shown that transgenic plants expressing the barley ribosome-inactivating gene have an increased resistance to fungal disease. 5

## II. Genes That Confer Resistance To A Herbicide, For Example:

(A) A herbicide that inhibits the growing point or meristem, such as an imidazalinone or a sulfonylurea. 10 Exemplary genes in this category code for mutant ALS and AHAS enzyme as described, for example, by Lee et al., *EMBO J.* 7: 1241 (1988), and Miki et al., *Theor. Appl. Genet.* 80: 449 (1990), respectively.

(B) Glyphosate (resistance imparted by mutant EPSP 15 synthase and *aroA* genes, respectively) and other phosphono compounds such as glufosinate (PAT and bar genes), and pyridinoxy or phenoxy propionic acids and cyclohexones (ACCase inhibitor-encoding genes). See, for example, U.S. Pat. No. 4,940,835 to Shah et al., which discloses the nucleotide sequence of a form of EPSP which can confer glyphosate resistance. A DNA molecule encoding a mutant *aroA* gene can be obtained under ATCC accession No. 39256, and the nucleotide sequence of the mutant gene is disclosed in 25 U.S. Pat. No. 4,769,061 to Comai. European patent application No. 0 333 033 to Kumada et al. and U.S. Pat. No. 4,975,374 to Goodman et al. disclose nucleotide sequences of glutamine synthetase genes which confer resistance to herbicides such as L-phosphinothricin. The nucleotide sequence of a phosphinothricin-acetyl-transferase gene is provided in European application No. 0 242 246 to Leemans et al. De Greef et al., *Bio/Technology* 7: 61 (1989), describe the production of transgenic plants that express chimeric bar genes coding for phosphinothricin acetyl transferase activity. Exemplary of genes conferring resistance to phenoxy propionic acids and cyclohexones, such as sethoxydim and haloxyfop, are the Acc1-S1, Acc1-S2 and Acc1-S3 genes described by Marshall et al., *Theor. Appl. Genet.* 83: 435 (1992).

(C) A herbicide that inhibits photosynthesis, such as a triazine (*psbA* and *gs+* genes) and a benzonitrile (nitrilase gene). Przibilla et al., *Plant Cell* 3: 169 (1991), describe the transformation of *Chlamydomonas* with plasmids encoding mutant *psbA* genes. Nucleotide sequences for nitrilase genes are disclosed in U.S. Pat. No. 4,810,648 to Stalker, and DNA molecules containing these genes are available under ATCC accession Nos. 53435, 67441 and 67442. Cloning and expression of DNA coding for a glutathione S-transferase is described by Hayes et al., *Biochem. J.* 285: 173 (1992).

## III. Genes That Confer Or Contribute To A Value-Added Trait, Such As:

(A) Nutritional enhancement, as illustrated by

(1) Higher lysine content: A cereal such as maize could be transformed with a gene that increases lysine content, making the cereal nutritionally more complete and thereby eliminating need for added lysine, for example, in poultry and swine feeds. 60

(2) Higher methionine content: A gene would be added to increase methionine levels in a cereal crop to offset an overall low methionine content, for example, in a poultry feed which combines lower- and higher-methionine components such as soybean and maize, respectively. 65

(B) Decreased phytate content

(1) Introduction of a phytase-encoding gene would enhance breakdown of phytate, adding more free phosphate to the transformed cereal. For example, see Van Hartingsveldt et al., *Gene* 127: 87 (1993), for a disclosure of the nucleotide sequence of an *Aspergillus niger* phytase gene.

(2) A gene could be introduced that reduces phytate content. This could be accomplished, for example, by cloning and then re-introducing DNA associated with the single allele which is responsible for maize mutants characterized by low levels of phytic acid. See Raboy et al., *Maydica* 35: 383 (1990).

(C) Modified carbohydrate composition effected, for example, by transforming maize with a gene coding for an enzyme that alters the branching pattern of starch. See Shiroza et al., *J. Bacteriol.* 170: 810 (1988) (nucleotide sequence of *Streptococcus mutans* fructosyltransferase gene), Steinmetz et al., *Mol. Gen. Genet.* 200: 220 (1985) (nucleotide sequence of *Bacillus subtilis* levansucrase gene), Pen et al., *Bio/Technology* 10: 292 (1992) (production of transgenic plants that express *Bacillus licheniformis*  $\alpha$ -amylase), Elliot et al., *Plant Molec. Biol.* 21: 515 (1993) (nucleotide sequences of tomato invertase genes), Sogaard et al., *J. Biol. Chem.* 268: 22480 (1993) (site-directed mutagenesis of barley amylase gene), and Fisher et al., *Plant Physiol.* 102: 1045 (1993) (maize endosperm starch branching enzyme II).

Synthesis of genes suitably employed in the present invention can be effected by means of mutually priming, long oligonucleotides. See, for example, Ausubel et al. (eds.), *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, pages 8.2.8 to 8.2.13 (Wiley Interscience 1990), and Wosnick et al., *Gene* 60: 115 (1987). Moreover, current techniques which employ the polymerase chain reaction permit the synthesis of genes as large as 1.8 kilobases in length. See Adang et al., *Plant Molec. Biol.* 21: 1131 (1993), and Bambot et al., *PCR Methods and Applications* 2: 266 (1993).

Maize lines that can be transformed via the present invention include, among others, inbreds that are employed in producing commercial hybrids. These inbreds, both proprietary and publicly-available, span many heterotic families. The preferred representatives within the heterotic groupings, and the relative use of entire heterotic patterns, vary with the market in question. For example, different germplasm is favored when breeding for the continental United States, including different geographic areas of adaptation (for example, the South, the East, the West, the North and the Central Corn Belt), for Europe and for South America, as well as for other international markets.

Callus-mediated methodology is unsuitable for many inbreds which do not produce the required callus response or 55 which provide callus that grows in a manner rendering the methodology unusably inefficient ("recalcitrant" inbreds). Accordingly, such methodology has been limited to a large extent to transformation of a few genotypes, such as, in maize, A188, A188 x B73, H99, Pa91, FR16 and genotypes obtained via a cross involving one of these genotypes. By contrast, meristem transformation pursuant to the present invention is applicable to any line, regardless of how that line responds to callus-mediated transformation. Thus, even cereal lines heretofore deemed recalcitrant to transformation can be transformed stably via the present invention. Illustrative of the maize inbreds thus affected are PHT47, PHP02, PHV78, PHK05, PHW20, PHR62, PHN37, PHM10, 65

PHV37, PHJ65, PHBW8, PHK29, PHJ33, PHP60, PHN73, and PHHV4. By the same token, the present invention should be applicable to newly-developed inbreds and to new heterotic groups which are created through the combination of existing germplasm, including "exotic" material brought into breeding programs from sources in the tropics and elsewhere.

According to a preferred embodiment, therefore, the present invention contemplates a transgenic plant that belongs to a cereal line that is recalcitrant to callus-based method transformation. Conversely, another preferred embodiment encompasses transgenic maize plants that are not produced by transformation of A188, A188 x B73, H99, Pa91 or FR16. In this context, the phrase "cereal line" denotes a group of gramineous plants of the sub-family Poaceae which display relatively little variation between individuals with respect to more than one distinctive trait, generally although not exclusively by virtue of several generations of self-pollination. (In addition, the term "line" here is used sufficiently broadly to include a group of plants vegetatively propagated from a single parent plant, via tissue culture techniques.) A plant is said to "belong" to a particular line if it (A) is a primary transformant ( $T_0$ ) plant regenerated from material of that line or (B) has a pedigree comprised of a  $T_0$  plant of that line. In this context, the term "pedigree" denotes the lineage of a plant, e.g., in terms of the sexual crosses effected such that a gene or a combination of genes, in heterozygous (hemizygous) or homozygous condition, imparts a desired trait to the plant.

The present invention is further described in the following examples, which are illustrative only. In carrying out the examples, a general procedure was followed for biolistic transformation. According to this procedure, 60 mg of 1.0 to 1.8  $\mu$ m tungsten microprojectiles (source: General Electric) were suspended in 2 ml of 0.1M  $HNO_3$  and sonicated for twenty minutes on ice. After centrifugation at 10,000 rpm to remove  $HNO_3$ , 1 ml of sterile deionized water was added, followed by a brief sonication and further centrifugation. This water rinse was repeated twice, after which the water was removed and 1 ml of 100% EtOH was added. The particles were resuspended by sonication, and the EtOH rinse was repeated. After the addition of 1 ml of sterile, deionized water and a further sonication, four aliquotes of the resulting suspension (250  $\mu$ l each) were pipetted into separate tubes (2 ml volume). Sterile, deionized water (750  $\mu$ l) was added to each tube, which then could be stored at  $-20^\circ C$ . For purposes of DNA preparation, 50  $\mu$ l of the sonicated tungsten microprojectile suspension were pipetted into a 1.5 ml tube, to which was added 1 to 10  $\mu$ g of the foreign DNA. After mixing, 50  $\mu$ l of 2.5 M  $CaCl_2$  solution were added and, with further mixing, 20  $\mu$ l of 0.1 M spermidine also were introduced. The resulting composition was mixed, sonicated, and then centrifuged for about ten seconds. After the supernatant was withdrawn and 250  $\mu$ l of 100% EtOH were added, the composition again was sonicated and centrifuged, and the supernatant was withdrawn. Finally, 30  $\mu$ l of 100% EtOH solution were added to the composition, which thereafter was used, in a 5  $\mu$ l aliquot per shot, with rupture disks ranging from 200–1100 p.s.i.

#### EXAMPLE 1

##### Transformation with Non-Lethal Selection

##### (A) Evaluation of Maize Histone Promoter Linked to NPTII

Ears of a proprietary maize genotype, designated "N10000" for purposes of this description, were harvested seven days after pollination at the early coleoptilar stage of development. Harvested ears were surface-sterilized in 50%

Chlorox with Tween 20 for 20 minutes, and then rinsed three times with sterile deionized water. Kernel tops were removed with a scalpel and embryos were dissected from endosperm. Sixty-seven embryos were placed axis side up, 10 embryos per plate, onto maturation medium (MS salts, 0.1 g/L myoinositol, MS vitamins, 0.5 mg/L zeatin, 150 g/L sucrose, and 6 g/L Sea-Kem agarose; pH 5.6 prior to autoclaving). Embryos were incubated overnight at  $28^\circ C$ . in the dark before bombardment.

In these studies, embryos were transformed with plasmids DP6212 and DP3953. DP6212 contains the 2xhistone-143 promoter, the first intron of the maize ADH1 gene, the nptII gene encoding neomycin phosphotransferase (NPTII), and a 3' transcript processing region from the Proteinase Inhibitor II (PinII) gene of potato. DP3953 contains the ubiquitin promoter, the first intron of the ubi gene, the gene encoding  $\beta$ -glucuronidase (GUS), and a 3' transcript processing region from the PinII gene. Embryos were bombarded with DP6212 and DP3953, mixed at a 1:1 ratio, at a concentration of one 1  $\mu$ g of total DNA per tube of acid-washed tungsten particles. This concentration, at ten times less than the standard, was optimal for yielding transformants with more uniform GUS staining patterns and yet having no detrimental effect on the function of the selectable marker or the frequency of transformation.

Pursuant to the above-discussed biolistics protocol, the particles were delivered as five 5- $\mu$ l shots per tungsten tube, using a PDS-1000 Helium gun with 1100 p.s.i. rupture disks. All embryos received one bombardment per plate.

After bombardment, embryos were maintained at  $28^\circ C$ . in the dark for seven days on Maturation Medium. Embryos then were transferred to 272K shoot elongation medium (MS salts, 0.1 g/L myoinositol, MS vitamins, 30 g/L sucrose, and 4 g/L gelrite) which contained 150 mg/L tobramycin sulfate as the selection agent. Embryos were incubated in the light at  $28^\circ C$ . At the time of transfer, embryos had elongated cotyledons.

At two, three and four weeks after bombardment, recovered plantlets were sampled and analyzed for GUS expression via methodology described by McCabe et al., *Bio/Technology* 87: 923-26 (1988). Leaf tips were placed in about 200  $\mu$ l of histochemical stain and allowed to incubate at  $37^\circ C$ . overnight in the dark to maximize GUS expression. Data from the first and second leaves are summarized in the following table.

TABLE 1

GUS Activity in Tissues of Transformed N10000			
Plate	#Plants Analyzed	#GUS +	GUS Sector Type
1	9	6	half leaves
2	8	5	half leaves or spotty
3	9	4	linear sectors
4	9	6	half leaves or spotty
5	7	3	linear sectors
6	11	2	complete staining

Any plantlets showing positive sectors were transferred to culture tubes containing shoot elongation medium that did not contain tobramycin. Each new leaf was examined for GUS expression. Plantlets that were consistently positive for GUS were transferred to the greenhouse for maturation when root development was well established. Plants that stopped expressing GUS were observed for phenotype changes, i.e., necrosis, bleaching or general lack of growth, which indicated that an escape from selection had occurred.

Plants with normal phenotypes were analyzed for NPTII protein by means of a NPTII ELISA kit available from 5'→3', Inc., 5603 Arapahoe Road, Boulder, Co. 80303 (catalog No. 5307-661-514). Positives were transferred to the greenhouse.

Transgenic plants maturing in the greenhouse were sampled for GUS activity or NPTII protein in each new leaf and in tassel and ear tissues to characterize the expression pattern in each plant. Pollinations were completed as selfs or as sibs. Eight to ten days after pollination, embryos were rescued by harvesting and surface sterilizing the ears, excising the embryos and placing the embryos on shoot elongation medium for germination. (This procedure was not required but was performed to accelerate the analysis process.) T1 leaf tissue was sampled for GUS histochemical assays and painted with 2% kanamycin sulfate in 0.2% SDS buffer to verify transmission of the transgenes. Samples of mature leaves were harvested from the T0 transformant for Southern analysis to further characterize the transformation.

Histochemical analysis of one N10000 plant, designated "2-4," demonstrated that GUS was expressed in leaves, silks (primary ear), primary ear husks and cob, and central spike and branches of tassel. In addition, Southern analysis confirmed the presence of the NPTII structural gene in leaf tissue harvested from the mature R<sub>0</sub> plant. Segregation of this hybridizing band correlated with NPTII-positive ELISA results in this plant. Analysis of the central stalk also showed GUS expression in epidermal layer of adventitious roots and in a vascular bundle of the central stalk.

#### (B) Transformation of Maize Lines with Non-Lethal Selection

Experiments were performed to determine the efficacy of non-lethal selection in a variety of maize genotypes. Genotype N10000 and several other proprietary genotypes, designated "P10000," "W20000," "E10000," "PHP02" and "R20000," respectively, for purposes of this description, were transformed with plasmids DP6212 and DP3953 as described above. Table 2 below enumerates data showing that the non-lethal selection method is applicable to various maize lines, based on co-transformation experiments where the expression of the second nonselected gene was used to assess stable sector frequency and size.

TABLE 2

Percentage of Plantlets Expressing GUS Activity After Germination on Nonlethal Selection Medium						
Experiment	N10000	P10000	W20000	E10000	PHP02	R20000
A	65.6	—	—	—	—	—
B	23.1	—	—	—	—	—
C	—	—	—	16.7	—	—
D	—	—	—	71.1	—	—
E	00.0	—	38.5	—	—	—
F	16.4	—	—	—	7.3	—
G	12.3	—	—	—	—	20.0
H	39.1	—	—	—	—	—
I	—	—	—	20.0	—	15.5
J	—	—	—	7.2	—	—
K	—	—	—	4.3	—	—
L	—	5.0	—	—	—	—
M	—	—	—	5.0	—	1.3
Averages	26.1	5.0	38.5	20.7	7.3	12.3

#### (c) Evaluation of Transformation Frequency When Meristem Reorganization Is Effected by Mechanical Disruption of Apical Dome Prior to Bombardment

Ears of genotypes E10000 and W20000 were harvested at the early coleoptilar stage of development and at 11 and 9 days, respectively, after pollination. One hundred and sixty

embryos were isolated and incubated on maturation medium, as described above.

The apical dome of several embryos was disrupted prior to bombardment to force the meristem to reorganize and form new meristematic areas. Mechanical disruption was performed by means of micromanipulation needles, ranging from 0.5 µm to 5 µm in diameter, which were attached to a World Precision Instruments M3301 micromanipulator. Needle penetration of each embryo was effected in the center of the apical dome to a depth ranging from a few microns to a few hundred microns, depending on the morphology of the embryo (embryos with a larger scutellum will tolerate deeper penetration). The preferred targeted depth of penetration was between 50 µm and 150 µm. Embryos then were bombarded with NPTII and GUS constructs, as described above.

Embryos were maintained in the dark at 28° C. for seven days on Maturation Medium and then transferred to 272K medium containing 150 mg/L Tobramycin sulfate. At time of transfer, embryos had multiple meristem formation with elongated cotyledons. Embryos were incubated in the light at 28° C.

At two, three and four weeks post-bombardment, first and second leaves of recovered plantlets were analyzed for GUS expression by histochemical assay. Table 3 shows the results of GUS assays, as well as observations on meristem formation.

TABLE 3

Effect of Mechanical Disruption on Transformation Efficiency and Meristem Formation			
Manipulation	N	# New Meristems	% GUS-Positive Plants
150 µm	80	89	45.9% [17/37 analyzed]
none	60	0	37.5% [3/8 analyzed]

These data demonstrate that the mechanical disruption of the apical dome resulted in new meristem formation and a higher frequency of transformation. In addition, mechanical disruption provided a more continuous GUS expression pattern in relation to non-manipulated plants, which displayed a narrower, more spotty pattern of GUS expression. Thus, meristems not disrupted frequently exhibited leaf tip GUS expression only, whereas most of the meristems that were disrupted showed wide, continuous sectors in leaves.

#### EXAMPLE 2

##### Transformation with Shoot Multiplication

##### (A) General Methodology

Embryos at the coleoptilar stage were isolated and cultured scutellum side down on an embryo maturation medium (10–20 embryos/plate). Since there can be considerable seasonal and genotypic variation affecting embryo ontogeny, embryo stage rather than size or days after pollination, was monitored carefully.

Embryos typically were matured on MS-based medium containing 0.5 mg/L zeatin, 1 mg/L indoleacetic acid, and elevated sugar levels which serve as an osmoticum. The embryos were cultured for a period ranging from 0–48 hours post-isolation, with 12–24 hours being optimal. Meristems then were bombarded with genes conferring kanamycin or streptomycin resistance, along with other nonselected genes, such as agronomic or visual marker genes.

After bombardment, the embryos were cultured in the dark to promote germination. After one to two weeks, the embryos were moved to a germination medium, such as hormone-free or low-hormone MS medium. The germinated plantlets generally had a swelling at the junction between the mesocotyl and epicotyl. This swelling occurred in the region containing the developing shoot meristem.

Two to three millimeter sections including the meristem were excised and cultured on a shoot proliferation medium which contained the appropriate hormones and a selection agent. The sections were regularly trimmed of elongated leaves and transferred to fresh medium every 10 to 14 days. Cultured meristems were incubated at 28° C. in the dark. After three to nine weeks, the proliferating meristems were transferred to an illuminated culture room.

Transformed sectors were identified one to two weeks after culture in the light, based on their green phenotype, i.e., nontransformed tissue remained bleached upon selection. In general, plants were regenerated by lowering the hormone concentration, although in some genotypes cytokinin concentrations were increased to promote plant regeneration. Since regenerated plants sometimes have difficulty rooting, rooting was promoted by culture on SH medium with 1 mg/l NAA, or by nicking the base of the stem and dipping the shoots in a 1 mg/ml NAA solution.

#### (B) NPTII Transformation of Honey and Pearl

One hundred eighty coleoptilar-stage maize embryos of the Honey and Pearl variety were harvested nine days after pollination. The scutella of the isolated embryos averaged 0.48 mm in length. These embryos were placed on embryo Maturation Medium (10 embryos per plate) and cultured overnight in the dark at 28° C.

Sixteen plates of the embryos were bombarded twice according to the above-described method with plasmid DP551, using 1.8 µm tungsten particles at a DNA concentration of 10 µg DNA/tube of tungsten. Plasmid DP551 contains ADH intron 1, GUS gene, and nos terminator, as well as ADH intron 1, NPTII gene, PinII terminator. Both GUS and NPTII genes are regulated by 35S CaMV sequences. Plates containing these embryos were cultured and matured in the dark at 28° C. Eight days later, a few of the embryos were placed in X-Gluc histochemical stain. All embryos contained intense blue staining, indicating GUS activity.

Most of the embryos had germinated nineteen days after particle bombardment. At this time, the region containing the meristem and leaf primordia was excised as described above and cultured on agar solidified MS medium with 2 mg/L BAP and 50 mg/L kanamycin. Leaf tissue was stained and chimeric blue staining sectors were observed in eight of the sixteen plates. The region containing the meristem was trimmed of elongated leaves and transferred to fresh medium every 10 to 14 days. Twenty six days after bombardment, the level of kanamycin was increased to 100 mg/L. Proliferating meristems were transferred to the light a week later. These experiments produced three independent transformation events. Two of the transformants have been characterized by PCR, GUS staining, NPTII ELISA assay and Southern analysis. One of these events exhibited strong GUS activity and high levels of NPTII protein. The T1 and T2 generations from this event were used for subsequent analysis. Progeny displayed a co-segregating, 1-to-1 ratio after outcrossing, based on both GUS activity and NPTII ELISA results (see Table 4) consistent with Mendelian inheritance of the integrated genes. Integration and segregation of the NPTII gene, which correlated with positive NPTII ELISA results, were demonstrated through Southern analysis of T<sub>1</sub> plants.

#### (C) aadA Transformation of Honey and Pearl

Coleoptilar stage Honey and Pearl embryos were isolated and cultured on 288B medium (MS medium with 0.5 mg/l zeatin, 1mg/l IAA, 0.25M sorbitol, and 4% sucrose solidified with 3 g/l gelrite). Eight plates with ten embryos per plate were bombarded once, as described above. Each particle preparation (enough for six shots) employed a combined total of 10 µg of DNA (5 µg DP4790+5 µg DP460 or DP3536). Plates 1 to 4 were bombarded with plasmid DP4790, which contains a 35S CaMV promoter, omega', aadA and ocs terminator (provided by Dr. Jonathan Jones, John Innes Institute), and with plasmid DP460, which contains a 35S CaMV promoter, ADH intron, GUS gene, and nos terminator. Plates 5 to 8 were bombarded with plasmids DP4790 and DP3536. The latter plasmid contains a cab promoter, ADH intron 6, GUS gene, and ocs terminator. All embryos were grown and germinated as described in part (B) of this example, supra. After germination, the regions containing the meristems were cultured on agar-solidified MS medium containing 2 mg/L BAP and 100 mg/L streptomycin sulfate.

After cultured meristems were moved to an illuminated culture room, a green sector was observed on a proliferating meristem on plate 6. All other cultured meristems were white due to streptomycin bleaching. GUS staining at this time revealed a mix of sectorized and non-sectorized blue staining leaves. About seven weeks after bombardment, sorting out was observed in the leaves from the transformation event on plate six. Some leaves were non-sectorized GUS+while others were still mericlinal. Transformation was confirmed using PCR, GUS staining and Southern analysis.

#### (D) Transformation of an Elite Inbred

Eight days after pollination, coleoptilar stage embryos of an elite inbred, designated "B30000" for purposes of this description, were isolated and cultured on 288L medium in fifteen plates containing twenty embryos per plate. Twelve plates were bombarded, using standard protocols. Briefly, particle bombardment was performed with six shots using 650 psi rupture disks and 1 µm tungsten particles, which were coated with plasmids DP5397 (proprietary agronomic gene) and DP5606 (Ubi promoter/Ubi-intron/NPTII/pin II terminator linked to cab promoter/ADH intron 6/GUS/ocs terminator) at a concentration of 5 µg DNA/particle preparation tube for each plasmid.

Plasmid DP5397 is a proprietary agronomic plasmid which contains a Bt gene, while plasmid DP5606 contains the Ubi promoter, Ubi intron, NPTII gene, and PinII terminator, which is linked to a cab promoter, ADH intron 6, GUS gene, and ocs terminator.

After bombardment the meristems were cultured on agar solidified MS medium containing 2 mg/L BAP, 0.25 mg/L, 2,4-dichlorophenoxy acetic acid and 3% sucrose. Five weeks after bombardment, meristems were placed on kanamycin selection (100 mg/L). To avoid irreversible bleaching of the meristems, this tissue was cycled between selective and non-selective media.

Five months after bombardment, a large green sector was removed from a bleached shoot culture. Three small leaves were removed from the sector and stained with X-Gluc. The leaves were found to express GUS activity in non-epidermal cells.

A single plant was regenerated from this series of experiments. The plant produced copious amounts of pollen and several ears. The pollen was found to be segregating for GUS expression, which was surprising since this gene was under the control of the cab promoter. All leaves of this plant exhibited strong, non-sectorized GUS activity. The tassel

glumes also were positive for GUS activity. Samples of leaf tissue from this T<sub>0</sub> plant contained the NPT-II and Bt proteins (as verified by their respective ELISA's) and exhibited strong GUS activity (fluorometric analysis). The GUS histochemical assay verified transmission to progeny in 42 of 106 seedlings sampled to date, which is consistent with Mendelian inheritance.

### EXAMPLE 3

#### Transformation Regimen Employing Immature Ear and Tassel Meristems

##### (A) Excision of Immature Ears

From plants harvested seven to nine weeks after planting, leaves were removed aseptically, one at a time, and the ears were exposed. The ears were dissected out of the husks under a dissecting microscope. Longitudinal bisection of the ears increased the response and exposed the meristems more fully to bombardment.

##### (B) Staging and Selection of Responsive Explants

The size of the whole excised ear and the developmental stage of the meristems were found to be reliable indicators of proper timing of harvest. Smaller ears are less developmentally determined and more responsive to hormonal stimuli, but fewer meristems survive resulting in fewer targets for transformation. Although smaller inflorescences have been used, two millimeters was used as the practical lower size limit for transformation experiments. The upper limit for selection of responsive targets was determined by meristem stage; developmental plasticity decreased dramatically once the glumes began to be obvious and approached the sides of the meristematic dome.

##### (C) Initial Culture Medium

Various media have been used, and inbreds respond differently to these variations. A preferred medium used in the initial stage of floral meristem culture (used for various genotypes) consisted of Murashige and Skoog salts, MS vitamins, 0.1 mg/l 2,4-D, 0.5 gm/l6-BAP, 1-proline at 12.2 μM, 8% sucrose, and silver nitrate at 30 mg/l. A preferred gelling agent is GELRITE (product of Merck and Co, Inc./Kelco division, Rahway, N.J.) at 3.5 g/l.

##### (D) Bombardment

Immature ear explants were bombarded using 650 psi rupture disks and a stainless steel screen (100 μm mesh size) suspended approximately 0.5 to 1.0 cm above the tissue. DNA precipitation and other bombardment parameters were as described in Example 1.

##### (E) Subsection, Subculture and Selection

Maintenance of rapid growth and survival of individual meristems was achieved by subsectioning the ears four to six days after isolation, into pieces with four to eight meristems each. These pieces were cultured onto shoot multiplication medium, which has the same basal composition as the initial culture medium (above) but with 1 mg/l BAP and 3% sucrose. Meristem tissue was subcultured repeatedly, at two week intervals on the shoot multiplication medium.

Incubation of bombarded ear meristems in X-gluc consistently resulted in high frequencies of transient GUS expression two days after bombardment. Stable sectors in leaves produced by multiple shoot clumps were found to express GUS one month after bombardment. At this stage, leaves were approximately 1 to 2 cm in length, and transformed sectors were found that extended more than half the length of the leaf. In addition, one meristem sacrificed at this stage expressed high levels of GUS in a histochemical assay.

One month of shoot multiplication was followed by one month of selection using 100 mg/l streptomycin. After this treatment, all material was subcultured once more onto

medium without the selective agent, and were additionally moved into the light. Leaves and shoots in non-selected cultures quickly turned green. Leaves in selected cultures remained bleached (white).

##### (F) Plant Regeneration

Putatively transformed shoots clumps were transferred to medium lacking plant growth regulators. Varying degrees of leaf development occurred on 1 mg/l BAP, and shoots soon formed and elongated in the absence of hormones.

##### (G) Rooting

Rooting at high frequency was effected via several days of exposure to MS- or SH-based media with 1-5 mg/l NAA.

### EXAMPLE 4

#### Transformation of Early Proembryo, Mid Proembryo, Late Proembryo, Transitional and Early Coleoptilar-Stage Embryos

Immature embryos at the mid proembryo, late proembryo, transitional and early coleoptilar stage were harvested and cultured on culture medium 610A, containing high concentrations of cytokinin and osmoticum. The 610A culture medium comprised MS salts, MS vitamins, 100 mg/l myo-inositol, 0.4 mg/l thiamine-HCl, 1 mg/l zeatin riboside, 0.1 mg/l BAP, 60 g/l sucrose, 400 mg/l asparagine, and 7 g/l Hazelton TC agar. After one day of recovery, the embryos were bombarded with DNA, by means of the particle gun as described above, and punctured in the center of the area of where the apical meristem will develop with a 0.5 μm micromanipulation needle.

Embryos were allowed to mature for 7 days in the dark and then transferred to a hormone-free medium containing 1 mg/l bialaphos. Following another 7 days of culture on hormone-free medium in the dark, the embryos were transferred to germination medium, and cultured in the light for continued germination. As leaves developed, plant phenotype was observed and samples were taken to check for sector formation by histochemical assay (GUS) as described above.

Healthy plants with normal phenotype and/or reporter gene activity were transferred to the greenhouse for maturation. The data shown in Table 4, which were generated via the above-discussed protocol, demonstrate for inbred N10000 the sector frequency obtained across several similar experiments, using embryos staged at mid proembryo, late proembryo, transitional, and early coleoptilar.

TABLE 4

Embryo Stage	Sector Frequency Obtained With Mid Proembryo, Late Proembryo, Transitional and Early Coleoptilar Stage Embryos				
	N	Trans- genes	GUS frequency	GUS pattern of expression	Sector Placement
Early Coleoptilar	328	BAR/GUS	14.2%	Leaf tips and files of 1-3 cells	Leaves 1 and 2
Transitional	250	BAR/GUS	22.5%	Saddle and Linear	Sectors start at Leaf 1, 5 or 11
Late Proembryo	200	BAR/GUS	34%	Saddle and Linear	Sectors start at Leaf 1 or Leaf 5
Mid Proembryo	110	BAR/GUS	3%	Linear and whole leaf	Sectors start at Leaf 1

The GUS frequency observed after targeting mid proembryos reflected, at the time these data were collected, a

relatively poor survival rate after bombardment and selection. But the addition of 1 mg/l zeatin to medium 610A, an increasing of the agar concentration (12 g/l), and the use of lower rupture disc pressure (200 p.s.i.) during particle delivery increases survival of mid proembryos after isolation and DNA delivery.

### EXAMPLE 5

#### Meristem Transformation—Direct Germination Approach

Genotype N10000 plants were pollinated and, eight days later, embryos were placed into culture. The harvested embryos thus were late-proembryo stage.

More specifically, embryos were cultured at day 0, axis up, onto modified 610A medium, containing 150 g/l sucrose, 1 mg/l zeatin, and 12 g/l agar, and incubated at 28° C. overnight in the dark. At day 1, following the overnight incubation, the apical meristems of all embryos were disrupted in the center of the apical dome using a 0.5 µm Femtotip micromanipulation needle. All embryos were returned for an overnight incubation at 28° C. in the dark. At day 2 bombardment was effected with the PDS-1000 Helium particle gun, one shot per plate, using 650PSI rupture disks. DNA employed in this regard was DP3528+DP3953 [2×35S::BAR+UBI::GUS] at 1 µg/tube of 1-µm tungsten. At day 2 all embryos were maintained in the dark at 28° C. for 7 days on 610A medium to allow meristem maturation to occur. At day 7 (after 7 days on 610A), embryos were transferred to 612 medium containing MS salts and vitamins, 0.001 mg/l kinetin, 0.1 mg/l adenine sulfate, 20 g/l sucrose, 6 g/l agar and 0.5 mg/L bialaphos, for germination and selection. At day 14 embryos were kept in the dark at 28° C. for 7 days before transfer to the light for further germination. On days 21–49 GUS histochemical assays on developing leaves were conducted, and on day 35 growing plantlets were transferred to tubes containing MS medium with no hormones and 5 mg/L bialaphos. On day 56 plant 6-1 (SID 180741) and plant 2-7 (SID 180742) were transferred to the greenhouse.

The total number of embryos cultured and bombarded was 48, of which 37 developed normally. The number of embryos that grew beyond leaf 1 was 17, with four plants showing GUS expression. Two plants survived 5 mg/L bialaphos selection with normal root development, and were transferred to the greenhouse for maturation.

SID 180741 and 180742 both showed GUS expression at the time of greenhouse transfer and had normal leaf and root development, whereas all other plants died. SID 180742 showed GUS expression in leaves 1–8 only.

TABLE 5

Sector Placement by GUS histochemical assay of SID180741	
Structure	GUS phenotype
Leaf 1	negative
Leaf 2	leaf tip
Leaf 3	leaf tip
Leaf 4	saddle sector: margin and midrib
Leaf 5	midrib sector
Leaf 6	saddle sector margin and midrib
Leaf 7	midrib sector
Leaf 8	saddle sector margin and midrib
Leaf 9	midrib sector
Leaf 10	saddle sector margin and midrib

TABLE 5-continued

Sector Placement by GUS histochemical assay of SID180741	
Structure	GUS phenotype
Leaf 11	midrib sector
Leaf 12	saddle sector margin and midrib
Leaf 13	midrib sector
Leaf 14	half leaf
Leaf 15	half leaf
Leaf 16	half leaf
Leaf 17	half leaf
Leaf 18	half leaf
Leaf 19	half leaf
Leaf 20	entire leaf
Leaf 21	entire leaf
Tassel	To date: central stalk shows pollen staining; 5 tassel branches also positive
Anthers	Endothecium and epidermis positive
Anther glumes	Epidermis positive

Leaves were painted with 1% Ignite in lanolin paste at the V6–V8 stage of development. SID 180741 showed resistance to Ignite in the sector (GUS expressing) areas only. SID 180742 showed no resistance to Ignite. PCR analyses were done on sampled leaves and confirmed presence of both GUS and BAR genes in SID 180741 and GUS genes in SID 180742.

One of the first major differences observed after moving towards an earlier developmental stage, i.e., when targeting late proembryos, was the production of saddle sectors (see Poethig (1986), supra, for a description). Extant information concerning meristem organization suggested to the present inventors that this might lead to germine transmission through the tassel. Saddle sectors extend from the leaf primordium up through the central portion of the apical dome and back into another portion of the leaf primordium. The extension of transgenic sectors into the central portion of the meristem greatly increases the probability of the sector contributing to the tassel and, ultimately, to pollen.

Previous research on maize anatomy and clonal analysis has shown that maize contains an organized, layered apical meristem beginning at the transitional stage of development. See Randolph, *J. Agric. Res.* 53: 881–916 (1936), and Poethig (1986), supra. Furthermore, a paper by Dawe and Freeling, *Developmental Biol.* 142: 233–45 (1990), regarding cell lineages in the male flower of maize, indicated that the L1 and L2 layers of the apical meristem give rise to the two layers of the anther wall. Only the inner layer is derived from the same cell lineage as the male germ cells, the L2. It also was found that events that occurred (by irradiation) before the organization of the shoot apical meristem contained sectors in both layers of the anther wall with inheritance through the pollen. Events that occurred after the transitional stage of development were limited to only one cell lineage with inheritance only when sectors occurred in the L2 layer.

Transformant 180741 was bombarded by the particle gun at the late proembryo stage of development, before meristem layer organization occurs. It contained a saddle sector which, by definition, is a sector which traverses the apical dome and bisects the meristem, in a region of the meristem that will later develop into the tassel (see Poethig (1986), supra). It also was wounded by a micro-manipulation needle, to encourage meristem reorganization, and exposed to bialaphos as the selective agent. GUS histochemical data showed expression in both layers of the leaves, in the anther wall, and in about 50% of the pollen from the central stalk.

## EXAMPLE 6

Stabilization of Transgenic Sectors by Means of  
Tillering

As noted above, tillering of transformed plants is an alternative to shoot multiplication for stabilization of transgenic sectors. Accordingly, elite lines may be induced to tiller, pursuant to the present invention, thereby stabilizing transgenic sectors.

In this example, tillering was induced in control plants using the method described by De Wolff, *Euphytica* 20: 524-26 (1971). A triangular incision was made with a number 11 scalpel blade at the approximate height of, or slightly above, the shoot apex of two week-old seedlings. The incision was made perpendicular to the plane of the leaves in order to avoid damage to the midribs. The shoot apex was removed from P10000, PHP02, G30000 and EI0000 seedlings. Each of these genotypes represents inbreds from significantly different heterotic families. Untreated plants of the same genotype were used as controls. If the incision was too far above the apex the procedure was repeated just below the initial incision.

The wounded plants and untreated controls were maintained in 24 hour continuous light (greenhouse during the day, growth chamber by night) for two weeks. A replicate treatment was grown under light/dark conditions.

Significant tillering was observed in the plants from which apices were removed. The influence of continuous light on tillering frequency, relative to normal light/dark conditions, was variable and may depend on genotype. The untreated controls did not tiller.

The hole made by the incision could have been plugged with lanolin and phytohormones, such as TIBA (1 mg/L) or BAP (10 mg/L), to increase tillering frequency. In the alternative, or in addition to the phytohormones, selective agents such as kanamycin could have been added to the incision to identify and select transgenic sectors.

What is claimed is:

1. A method for producing transgenic cereal plants that will transmit introduced DNA to progeny, comprising the steps of

(A) introducing foreign DNA into target cells selected from the group consisting of (i) cells of a meristem that is not enclosed by primordial sheathing leaves and (ii) cells that contribute to said meristem; then

(B) inducing reorganization of said meristem to increase transgenic sector size, whereby the likelihood that a transgenic sector will contribute to germline transmission is increased, wherein said reorganization is effected through at least one manipulation selected from the group consisting of (i) imposition of a non-lethal selective pressure on said meristem, (ii) mechanically-induced meristem reorganization, and (iii) hormonally-induced shoot multiplication combined with nonlethal selective pressure; and thereafter

(C) exposing said meristem to conditions under which it differentiates to form a plantlet, wherein said plantlet contains said transgenic sector or is homogeneously transformed by said foreign DNA, such that said plantlet can be grown into a transformed cereal plant that will transmit said foreign DNA to progeny.

2. A method according to claim 1, wherein step (A) is effected at early proembryo, mid proembryo, late proembryo, transitional or early coleoptilar stage.

3. A method according to claim 1, wherein said foreign DNA is introduced into a plurality of meristems, and at least some of said meristems differentiate in step (C) to form a plurality of plantlets.

4. A method according to claim 1, wherein said conditions in step (C) are such that said meristems undergo maturation and plant differentiation to form shoot apices, and wherein said method further comprises effecting reorganization of meristem tissue in said shoot apices to enlarge transformed sectors or to produce periclinal L2 chimeras.

5. A method according to claim 4, wherein said effecting of reorganization comprises exposing said shoot apices to nonlethal selection pressure such that transformed cells have a competitive growth advantage over nontransformed cells in said shoot apices, and the proportion of transformed cells in said shoot apices is increased.

6. A method according to claim 1, where step (A) comprises biolistic bombardment of an embryo at a developmental stage no later than the coleoptilar stage.

7. A method according to claim 4, wherein said effecting of reorganization comprises culturing said shoot apices under conditions such that multiple shoots proliferate therefrom through organogenesis.

8. A method according to claim 1, further comprising a step before step (C) of wounding said apical dome selectively.

9. A method according to claim 8, wherein said wounding is carried out before step (A).

10. A method according to claim 1, wherein step (A) comprises biolistic bombardment of meristems on immature ear or tassel explants.

11. A method according to claim 1, wherein said target cells are maize, sorghum, wheat, barley, oat or rice target cells.

12. A method according to claim 11, wherein said target cells are maize target cells.

13. A method according to claim 4, further comprising the steps of (i) dissecting out of an axillary bud from above the base of a leaf of a plantlet when a chimeric sector is observed in a substantial portion of said leaf, and then (ii) germinating said axillary bud into a whole plant or subjecting said axillary bud to shoot multiplication.

14. A method according to claim 1, further comprising the steps of (i) removing the shoot apex from seedlings of said plantlet, whereby a wound is produced, then (ii) growing said plantlets to induce formation of a plurality of tillers, and (iii) selecting a transgenic tiller from said plurality.

15. A method according to claim 14, wherein phytohormones that increase tillering are introduced, subsequent to step (i), into said wound.

16. A method according to claim 14, wherein a selective agent is introduced after step (i) into the wound, to identify and select transgenic sectors.

17. A method according to claim 1, wherein said nonlethal selective pressure is provided by a selective agent selected from the group consisting of kanamycin, streptomycin, hygromycin, norflurazon and bialaphos.

\* \* \* \* \*

## **EXHIBIT C**

## Genetic Transformation of Wheat Mediated by *Agrobacterium tumefaciens*

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A rapid *Agrobacterium tumefaciens*-mediated transformation system for wheat was developed using freshly isolated immature embryos, precultured immature embryos, and embryogenic calli as explants. The explants were inoculated with a disarmed *A. tumefaciens* strain C58 (ABI) harboring the binary vector pMON18365 containing the  $\beta$ -glucuronidase gene with an intron, and a selectable marker, the neomycin phosphotransferase II gene. Various factors were found to influence the transfer-DNA delivery efficiency, such as explant tissue and surfactants present in the inoculation medium. The inoculated immature embryos or embryogenic calli were selected on G418-containing media. Transgenic plants were regenerated from all three types of explants. The total time required from inoculation to the establishment of plants in soil was 2.5 to 3 months. So far, more than 100 transgenic events have been produced. Almost all transformants were morphologically normal. Stable integration, expression, and inheritance of the transgenes were confirmed by molecular and genetic analysis. One to five copies of the transgene were integrated into the wheat genome without rearrangement. Approximately 35% of the transgenic plants received a single copy of the transgenes based on Southern analysis of 26 events. Transgenes in  $T_1$  progeny segregated in a Mendelian fashion in most of the transgenic plants.

In the early 1980s, the era of plant transformation was initiated when *Agrobacterium tumefaciens*-mediated gene delivery was reported for the production of transgenic plants (De Block et al., 1984; Horsch et al., 1984, 1985). Initial successes were limited to the Solanaceae, tobacco in particular. This dramatically changed throughout the 1980s and into the 1990s, and it is now possible to transform a wide range of plants, including many agronomically important crops such as soybean, cotton, peanut, and pea (Hinchee et al., 1988; Umbeck et al., 1989; Schroeder et al., 1993; Cheng et al., 1996). Although *A. tumefaciens*-mediated transformation has significant advantages over naked DNA delivery, such as introduction of a few copies of genes into the plant genome, high co-expression of introduced genes, and easy manipulation in vitro, the *A. tumefaciens*-mediated transformation method for gene transfer has been limited to dicotyledonous plants (Songstad et al., 1995).

Several reports presented early attempts to transform the Gramineae with *A. tumefaciens*, including *A. tumefaciens*-mediated infection of plants with viral genomes (Grimsley

et al., 1988; Raineri et al., 1990; Gould et al., 1991; Mooney et al., 1991; Chan et al., 1992, 1993; Schläppi and Hohn, 1992; Shen et al., 1993). Chan et al. (1993) first reported the production of transgenic rice plants by inoculating immature embryos with an *A. tumefaciens* strain and proved the transformation by molecular and genetic analysis. Recently, significant progress was made in *A. tumefaciens*-mediated transformation of rice and maize: a large number of transgenic plants were regenerated and characterized (Hiei et al., 1994; Ishida et al., 1996). Convincing and unambiguous data on transgene expression, gene segregation in the progeny, and DNA analysis were presented in these papers.

There have been limited studies on *A. tumefaciens*-mediated transformation of wheat (*Triticum aestivum* L.). Hess et al. (1990) pipetted *A. tumefaciens* into the spikelets of wheat, and several kanamycin-resistant grain progeny were obtained. However, the protocol was not reproducible and the Southern hybridization was not convincing in this study. Deng et al. (1990) infected the base of the leaf sheath and spike stem of wheat plants with several wild-type *A. tumefaciens* strains and opine-synthesizing tumors formed from these tissues. Mooney et al. (1991) infected the immature embryos of wheat with *A. tumefaciens* and a few kanamycin-resistant colonies were generated.

Here we present an *A. tumefaciens*-mediated transformation method for wheat using freshly isolated immature embryos, precultured immature embryos, and embryogenic calli as explants. We produced a large number of transgenic plants and demonstrated stable integration, expression, and inheritance of transgenes in wheat plants.

### MATERIALS AND METHODS

#### Stock Plants and Explant Tissues

A spring wheat, *Triticum aestivum* cv Bobwhite, was used throughout this study. Stock plants were grown in an environmentally controlled growth chamber with a 16-h photoperiod at  $800 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by high-intensity discharge lights (Sylvania, GTE Products Corp., Manchester, NH). The day/night temperatures were 18/16°C. Immature caryopses were collected from the plants 14 d after anthesis. Immature embryos were dissected aseptically and cultured on a semisolid or liquid CM4 medium (Zhou et al., 1995) with  $100 \text{ mg L}^{-1}$  ascorbic acid (CM4C).

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Abbreviations: MS, Murashige-Skoog; T-DNA, transfer-DNA.

The MS salts (Murashige and Skoog, 1962) in the CM4C medium were adjusted to full strength (the original amounts) or one-tenth-strength (Fry et al., 1987). The immature embryos were cultured on these media for 3 to 4 h (freshly isolated) or 1 to 6 d (precultured). Embryogenic calli were prepared by culturing the immature embryos on CM4C medium for 10 to 25 d. The callus pieces derived from immature embryos were inoculated with *A. tumefaciens* without being broken down (intact), or only the embryogenic callus sectors were selected and separated into small pieces (approximately 2 mm).

#### A. *tumefaciens* Strain, Plasmid, and Culture

Disarmed *A. tumefaciens* C58 (ABI) harboring binary vector pMON18365 (Fig. 1) was used for all the experiments. pMON18365 contains the GUS (*uidA*) gene with an intron and the NPT II gene as a selectable marker within the T-DNA region. Each gene was under the control of an enhanced 35S (E35S) promoter. Cultures of *A. tumefaciens* were initiated from glycerol stocks and grown overnight at 25 to 26°C with shaking (150 rpm) in liquid Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing 50 mg L<sup>-1</sup> kanamycin, streptomycin, spectinomycin, and 25 mg L<sup>-1</sup> chloramphenicol with 200 μM acetosyringone, to mid-log phase (OD<sub>660</sub> = 1–1.5). The *A. tumefaciens* cells were collected by centrifugation and re-suspended in liquid inoculation medium (CM4C with one-tenth-strength MS salts and supplemented with 10 g L<sup>-1</sup> Glc and 200 μM acetosyringone). The *A. tumefaciens* cell density was adjusted to give an A<sub>660</sub> of 1 to 2 for inoculation.

#### Inoculation and Co-Cultivation

The immature embryos and embryogenic calli maintained on the CM4C medium as described above were transferred into an *A. tumefaciens* cell suspension in Petri dishes. A surfactant (0.01–0.075% [v/v] Silwet, Monsanto, St. Louis, MO) or pluronic F68 (0.01–0.2% [w/v] Sigma) was added to the inoculation medium in some experiments. The inoculation was conducted at 23 to 25°C for 3 h in the dark. After inoculation the *A. tumefaciens* cells were removed by vacuum or with a transfer pipette, and the explants were placed on semisolid or on a filter paper wetted with liquid CM4C with one-tenth-strength or full-strength MS salts and supplemented with 10 g L<sup>-1</sup> Glc and 200 μM acetosyringone. The co-cultivation was performed at 24 to 26°C in the dark for 2 or 3 d.

#### Selection and Regeneration of Transgenic Plants

After co-culture the infected immature embryos and calli were cultured on the solid CM4C medium with 250 mg L<sup>-1</sup>

carbenicillin for 2 to 5 d without selection. *A. tumefaciens*-infected explants were then transferred to CM4C medium supplemented with 25 mg L<sup>-1</sup> G418 and 250 mg L<sup>-1</sup> carbenicillin for callus induction. Two weeks later, the explants were transferred to the first regeneration medium, MMS0.2C (consisting of MS salts and vitamins, 1.95 g L<sup>-1</sup> Mes, 0.2 mg L<sup>-1</sup> 2,4-D, 100 mg L<sup>-1</sup> ascorbic acid, and 40 g L<sup>-1</sup> maltose, solidified by 2 g L<sup>-1</sup> gelrite) supplemented with 25 mg L<sup>-1</sup> G418 and 250 mg L<sup>-1</sup> carbenicillin.

At transfer to the regeneration medium, each piece of callus derived from one immature embryo or one piece of inoculated callus was divided into several small pieces (approximately 2 mm). In another 2 weeks, young shoots and viable callus tissues were transferred to the second regeneration medium, MMS0C, which contains the same components as MMS.2C with all antibiotics except 2,4-D included. When the shoots developed into about 3-cm or longer plantlets, they were transferred to larger culture vessels containing the second regeneration medium for further growth and selection. Leaf samples were taken from some of the plantlets for the GUS histochemical assay at this stage. Plants that were highly G418 resistant or GUS positive were transferred to soil. All of the plants derived from the same embryo or piece of callus were considered to be clones of a given event.

#### GUS Histochemical Assay

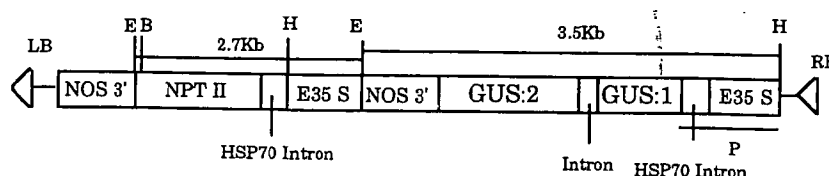
GUS activity was assayed histochemically in a 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution using the buffer described by Jefferson (1987) except that 20% methanol was added to eliminate the endogenous GUS activity.

#### Functional Assay of NPT II Genes

##### Paromomycin Spray

T<sub>1</sub> seeds harvested from each T<sub>0</sub> plant were planted in 2-inch pots grown under the same conditions as the stock plants as described above. When plants reached the 3-leaf stage, they were sprayed with 2% (w/v) paromomycin (Sigma) plus 0.2% (v/v) Tween 20. One week later the plants were evaluated for paromomycin damage. The plants with a functional NPT II gene showed no bleached spots, whereas the plants without a functional NPT II gene exhibited bleached spots throughout. Paromomycin was used in this assay and the leaf-bleach assay as described in the following section because it is a similar aminoglycoside antibiotic to G418, and is more effective and less expensive for these assays.

**Figure 1.** T-DNA regions of pMON 18365. RB, Right border; LB, left border; E35S, enhanced 35S promoter; HSP 70 intron, maize heat-shock protein 70 gene intron; NOS-3', 3' signal of nopaline synthase; NPT II, neomycin phosphotransferase II; H, *Hind*III; E, *Eco*RI; B, *Bam*HI; P, probe.



### Leaf-Bleach Assay

After the  $T_0$  plants were established in soil, leaf samples (5–7 mm long) were taken from the youngest fully expanded leaves and placed in a 24-well culture plate (Costar, Cambridge, MA). Each well was filled with 0.5 mL of a water solution composed of 300 mg L<sup>-1</sup> paromomycin and 100 mg L<sup>-1</sup> fungicide (Benlate, DuPont) or 100 mg L<sup>-1</sup> fungicide only. Three leaf samples taken from the same leaf of each plant were placed in two wells containing paromomycin and fungicide and one well containing fungicide only, respectively. Leaf samples from the nontransformed cv Bobwhite plants at a similar developmental stage were used as a negative control. The samples were vacuum-infiltrated in a desiccator using an in-house vacuum system for 5 min and then the plates were sealed with Parafilm before being placed under light for 3 d. The leaf samples that were highly resistant to paromomycin remained green in most of the area except around the edges (<1 mm wide), indicating that the NPT II gene was functional. The leaf samples from the plants without the gene or with a non-functional gene were bleached completely by paromomycin (as were the negative controls) (Fig. 2D) or had only small patches of green areas.

### DNA Analysis

Genomic DNA was isolated from leaf tissue of  $T_0$  plants and  $T_1$  progeny following the method of Roger and Bendich (1985). An equal amount of EcoRI-digested genomic DNA (15 µg per lane) was separated on an agarose gel, blotted onto a membrane, and probed with a <sup>32</sup>P-labeled fragment containing the enhanced 35S promoter and the 5' intron of the heat-shock protein 70 gene from maize following the manufacturer's protocol for the GeneScreen Plus membrane (DuPont).

### Progeny Analysis

The segregation of the GUS and NPT II genes in the progeny of  $T_1$  or reciprocal crosses was determined by one of the following methods: (a) paromomycin spray on the  $T_1$  seedlings and GUS histochemical assay on leaf tissue, as described above; (b) leaf-bleach assay on the  $T_1$  seedlings at the two-leaf stage; and (c) GUS histochemical assay on the immature (17 d after anthesis or older) and mature seeds harvested from the  $T_0$  plants. The immature seeds were sterilized in 10% (v/v) bleach (containing 5.25% sodium hypochlorite) for 15 min followed by three rinses with sterile water. The mature seeds were soaked in the water for several hours and then sterilized in 20% bleach for 40 min. Finally, the seeds were washed in sterile water three times for 30 min each. Each seed was longitudinally cut into two uneven parts. The embryo from the large part was isolated and cultured on the MMSOC medium for germination. The seedlings were eventually transferred to soil. The small part and the large part without the embryo were used for the GUS histochemical assay. The  $T_1$  seeds with or without the functional GUS gene could be determined based on the GUS activity in the embryo and endosperm

tissues (Fig. 2H). The plants in soil were also assayed by leaf-bleach assay for the NPT II activity and GUS histochemical assay at different stages. The data were then analyzed by the  $\chi^2$  test to determine the number of the functional GUS or NPT II gene loci.

## RESULTS

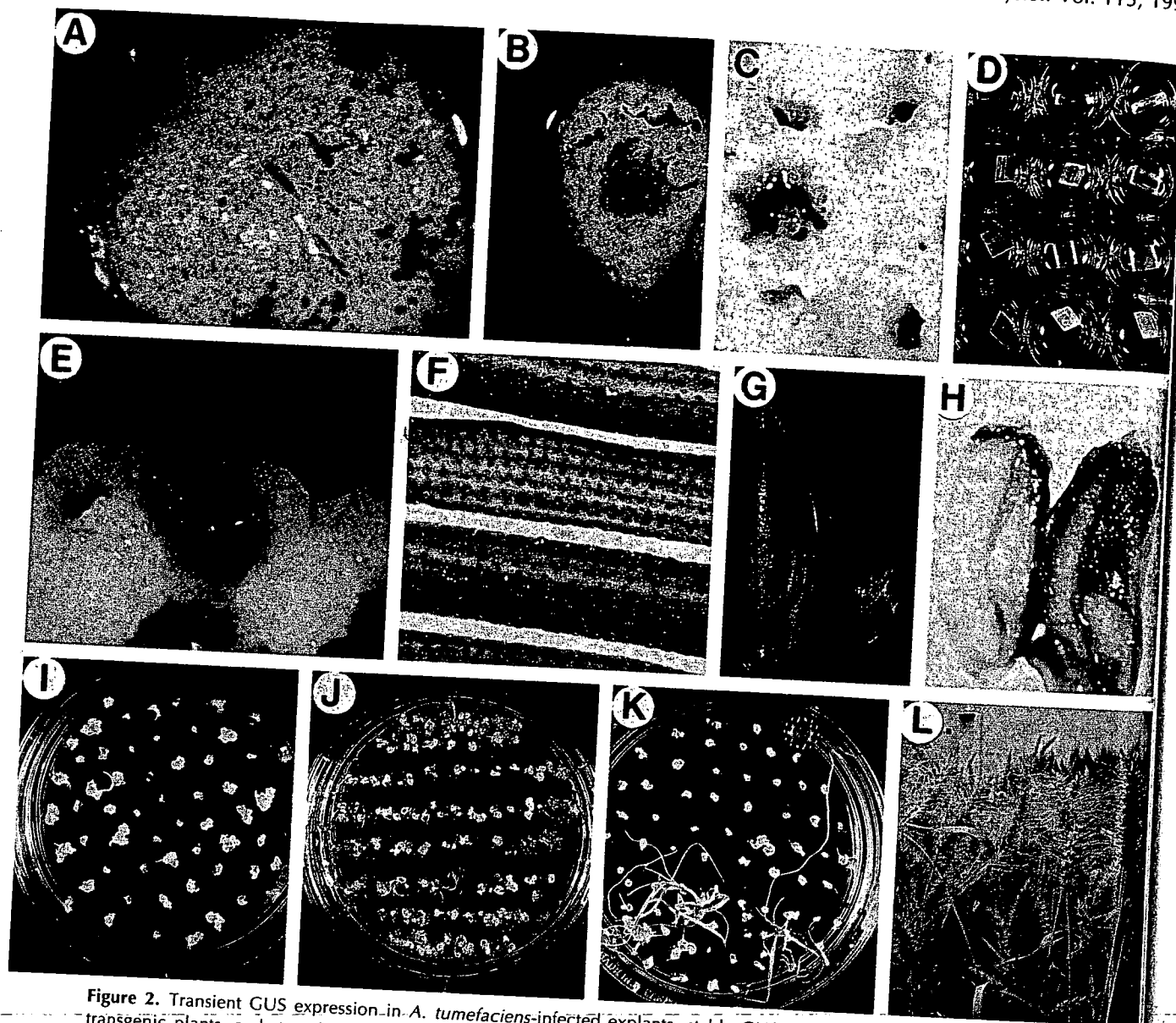
### Factors Influencing the Efficiency of T-DNA Delivery

Various factors influencing the efficiency of T-DNA delivery were evaluated in the preliminary experiments. These factors include different explant types, *A. tumefaciens* cell density for inoculation, inoculation and co-culture time period, co-culture medium, surfactants in the inoculation medium, and induction agents in the inoculation and co-culture media. Leaf tissue from young seedlings, immature inflorescences, freshly isolated immature embryos, or precultured immature embryos, embryogenic callus derived from immature embryos, and cells in suspension cultures derived from wheat cv Mustang were inoculated and co-cultured with *A. tumefaciens* ABI:pMON18365.

GUS expression was detected in all of the tissues after either 2 or 3 d of co-culture and a 2-d delay of selection. Highly efficient T-DNA delivery was observed on both freshly isolated immature embryos and precultured immature embryos when surfactant (Silwet) was present in the inoculation medium (Fig. 2, A and B). The GUS spots were present across all of the scutellum surface of freshly isolated immature embryos, whereas most of the GUS spots were localized on the areas starting to form callus in the precultured immature embryos. Leaf sections, when vacuum infiltration was applied during inoculation, showed high GUS activity. The suspension cells exhibited the highest-efficiency T-DNA delivery even without the addition of surfactant in the inoculation medium. Therefore, the suspension cells were chosen as a model system to optimize the transformation parameters for wheat (M. Cheng, J.E. Fry, C.M. Hironaka, and T.W. Conner, unpublished data).

A moderate number of GUS spots were observed on the embryogenic callus, whereas the spots were usually larger than in the immature embryos. Higher *A. tumefaciens* cell density and a longer time for inoculation and co-culture usually yielded more efficient T-DNA delivery on various tissues or cells, but more cell damage was observed. The salt strength in the inoculation medium was also found to influence the T-DNA delivery. For example, when one-tenth-strength MS salts were used for the inoculation and co-culture medium, transient GUS expression was significantly higher on the freshly isolated immature embryos than when the full-strength MS salts were used.

Another significant factor influencing T-DNA delivery was the presence of a surfactant in the inoculation medium. Two types of surfactants were evaluated based on T-DNA delivery efficiency with different wheat tissues. Both Silwet and pluronic F68 were found to have a significant positive effect on the transient GUS expression on different explants, especially on the immature embryos (Table I; Fig. 2A). Silwet at 0.01% started to enhance the transient GUS



**Figure 2.** Transient GUS expression in *A. tumefaciens*-infected explants, stable GUS expression in various tissues from transgenic plants, and steps in the regeneration of transgenic plants. A, Transient GUS expression in a freshly isolated immature embryo 4 d after inoculation. B, Transient GUS expression in a precultured immature embryo 3 d after inoculation. C, Transient GUS expression in embryogenic calli 5 d after inoculation. D, Leaf-bleach assay. The wells of the first column (left) contained 100 mg L<sup>-1</sup> fungicide-water solution and the remaining wells contained the 300 mg L<sup>-1</sup> paromomycin and 100 mg L<sup>-1</sup> fungicide-water solution. The first three rows of wells included leaf samples from three transgenic events with functional NPT II activity. The last column (bottom) was a leaf sample from a nontransgenic plant as a control. E, GUS expression in stably transformed, embryo-like tissue. F, GUS expression in young ovary and glume tissues of a transgenic plant. G, GUS expression in young leaf tissue from a transgenic plant. H, Segregation of the GUS expression in T<sub>1</sub> seeds assayed at 20 d after anthesis from a GUS-positive T<sub>0</sub> plant. Some seeds showed GUS activity in both the pericarp (the maternal tissue) and the aleurone layer (right), and others had GUS activity only in the pericarp (left). I, Callus induction on G418-containing CM4C medium. Inoculated immature embryos were cultured on selective callus-induction medium CM4C for 2 weeks. J, Shoot regeneration from embryogenic calli after 2 weeks of culture on first-regeneration medium MMS.2C containing G418. K, Plantlet regeneration after the embryogenic calli or shoots were cultured on second-regeneration medium MMS0C containing G418 for 2 weeks. L, Transgenic T<sub>0</sub> plants set seeds in a growth chamber.

expression on the scutellum side of the embryos. The concentration of Silwet at 0.05% gave the highest transient GUS expression, approximately 19-fold higher than the control. However, when the concentration of Silwet was

greater than 0.05%, most of the immature embryos could not survive. Based on this result, 0.01 to 0.02% Silwet was used routinely in our stable transformation experiments. Pluronic F68 at 0.01 to 0.05% had the same effect as Silwet

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**Table I.** Effect of surfactant when present in the inoculation medium on transient GUS expression in freshly isolated immature embryos (IE)

Concentration of Surfactant (Silwet)	IEs with GUS Spots	GUS Spots/IE
% (v/v)	% of total	
0.00	11/34 (34)	7.8
0.01	15/19 (79)	17
0.05	13/13 (100)	149
0.1	8/8 (100)	111
0.5	4/4 (100)	140

on the transient GUS expression on the immature embryo explants. Although both Silwet and pluronic F68 enhanced the efficiency of T-DNA delivery on the precultured immature embryos and embryogenic calli, they were not as significant as in the immature embryos. Silwet and pluronic F68 at 0.02% increased the transient GUS expression approximately 4-fold in the embryogenic calli compared with the control. An average of 30 blue spots was observed on each embryogenic callus (14 d old, intact) (Fig. 2C).

The presence of induction agents such as acetosyringone and Glc in the inoculation and co-culture media was crucial for efficient T-DNA delivery on some of the explants. For example, when acetosyringone and Glc were absent in the inoculation and co-culture media, the T-DNA delivery efficiency was significantly reduced in the freshly isolated immature embryos.

#### Regeneration of Transgenic Wheat Plants from Various Explants

*A. tumefaciens*-infected immature embryos and embryogenic calli were cultured on callus-induction medium CM4C with G418 for selection. Two weeks after callus induction, approximately 30 to 80% of the immature embryos formed embryogenic callus (Fig. 2I), whereas the inoculated embryogenic calli proliferated further on this medium. The GUS assay on some of the explants at this stage showed that the transformed, embryo-like tissue had developed from some of the inoculated explants (Fig. 2E). Developed calli were then broken down into small pieces, and transferred to the first regeneration medium for further selection. Multiple green shoots (most of them were not transformed) regenerated rapidly from the embryogenic calli (Fig. 2J). After 2 weeks of selection on the first regeneration medium, all of the viable shoots and callus tissues were transferred to the second regeneration medium, MMSOC with G418, for further selection (Fig. 2K). On this medium the most likely transformed shoots showed high resistance to G418, whereas most of the non-transformed shoots were not able to grow rapidly. Finally, highly resistant plantlets were transferred to larger culture vessels for further growth and selection. The transformed plants usually grew vigorously and formed strong root systems on the G418-containing MMSOC medium. The plants that survived the selection were moved to soil when they were approximately 10 to 15 cm in length.

#### Identification of Transgenic Plants and Transformation Efficiency

Most of the transgenic plants were identified by the GUS assay on the leaf tissues while the plantlets grew in the regeneration medium. After they were moved to soil, different tissues were collected at various stages for additional histochemical GUS assay. Leaf samples were also collected after the plants survived in soil for the leaf-bleach assay. Most of the plants had visible GUS activity in different tissues (Fig. 2, F-H), although the younger leaf tissue had higher activity than the older tissue, and young floral tissue had higher activity than the leaf tissue. However, a few of the plants that showed no visible GUS activity in leaf tissue had relatively high GUS activity in young floral tissues such as young ovary, stigma, glume, and lemma.

All of the plants showing GUS expression also had NPT II activity determined by the leaf-bleach assay except one that showed high-NPT II activity but no detectable GUS activity in any of the tissues (Table IV, event 21). The co-expression of GUS and NPT II genes in the plants produced via *A. tumefaciens*-mediated transformation was over 98% (49/50, Table IV) in our study. In contrast, the co-expression of the gene of interest (including the GUS gene) and the NPT II gene in the plants generated using the biolistic method, with either co-bombardment or 2 genes in the same construct, was from 42 to 62% in our laboratory, based on the analysis of 343 events with 4 different genes of interest. Therefore, the co-expression of two genes in the transgenic plants was significantly higher with the *A. tumefaciens*-mediated transformation than with the biolistic method.

Transgenic plants produced from all three kinds of explants are summarized in Table II. The transformation efficiencies for the freshly isolated immature embryos, precultured immature embryos, and embryogenic calli were  $1.12\% \pm 0.79$  ( $\bar{X}\% \pm SE$ ),  $1.56\% \pm 1.19$ , and  $1.55\% \pm 1.08$ , respectively, no significant difference in the transformation efficiency was shown among the three explant types, although it varied among experiments. Transgenic plants could be regenerated from all three explants; however, several experiments with all three explants actually failed to produce any transgenic plants. These experiments were not included in Table II. The freshly isolated immature embryos always showed efficient transient GUS expression when the surfactants were present in the inoculation medium, but they could not recover well after inoculation and co-cultivation.

Although many different media and co-culture conditions were attempted, the majority of the inoculated immature embryos failed to form embryogenic calli or formed very limited calli on the scutellum surface. Precultured embryos usually showed good transient GUS expression on areas starting to form callus, and also exhibited better culture response. Among these three explants, embryogenic callus cultured for more than 10 d in the callus-induction medium showed the best culture response. Usually, 100% of the explants continued to proliferate on the callus-induction medium with the selection agent present.

**Table II.** Summary of transformation results using three kinds of explants

Experiment	Explant <sup>a</sup>	Explants (A)	Transgenic Events (B)	Transformation Efficiency (B/A)
		no.		%
1	FIIE	160	1	0.6
2	FIIE	250	3	1.2
3	FIIE	700	1	0.14
4	FIIE	124	1	0.8
5	FIIE	140	2	1.4
6	FIIE	38	1	2.6
7	PCIE (1 d)	23	1	4.3
8	PCIE (3 d)	98	1	1.0
9	PCIE (3 d)	104	2	1.9
10	PCIE (3 d)	36	1	2.8
11	PCIE (5 d)	97	1	1.0
12	PCIE (6 d)	40	1	2.5
13	EC (10 d)	239	1	0.4
14	EC (10 d)	232	1	0.4
15	EC (14 d)	47	1	2.1
16	EC (15 d)	110	3	2.7
17	EC (17 d)	50	1	2.0
18	EC (21 d)	73	2	2.7
19	EC (25 d)	308	1	0.3

<sup>a</sup> FIIE, Freshly isolated immature embryo; PCIE, precultured immature embryo; EC, embryogenic callus. The number of days of the immature embryos cultured on callus induction medium (CM4C) prior to inoculation is given in parentheses.

### Characterization of the T<sub>0</sub> Plants

Plants identified as transgenic were grown in a growth chamber and evaluated for morphology and fertility. More than 100 events were established in soil and examined (Fig. 2L). All of the plants were fertile or partially fertile. The majority (about 80%) of the transformed plants produced as many seeds as the seed-derived control plants.

DNA was extracted from leaf tissue of 26 T<sub>0</sub> plants derived from independent events, and digested with *Eco*RI and hybridized with a probe consisting of E35S and the maize HSP 70 intron sequence (Fig. 3). DNA from non-transformed plants used as a negative control showed no hybridization to the probe. Since the T-DNA of

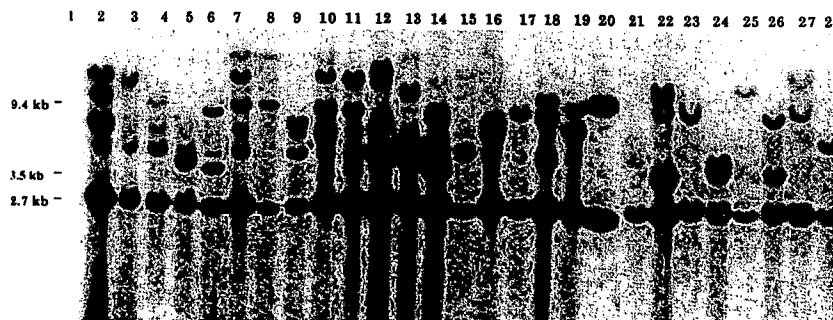
pMON18365 had two *Eco*RI sites, the 2.7-kb band represented the internal fragment with the NPT II gene cassette without nos 3' (Fig. 1). All 26 transgenic events had the 2.7-kb band (Fig. 3; Table III).

Because the third *Eco*RI site must be derived from the wheat genome, the number of hybridizing bands around or greater than 3.5 kb reflected the number of copies of the integrated gene (GUS) in the plants unless repeats of multiple copies of the T-DNA had been integrated. All of the detected bands except the 2.7-kb band represented the fragments of more than 3.5 kb. The mobilities of the bands differed from plant to plant, indicating independent events and random integration. The copy number of the integrated gene (GUS) varied from 1 to 5 (Fig. 2; Table III). A single copy of the transgene (GUS) was carried by 35% of the plants (9/26), and 50% (13/26) contained two or three copies. Only 15% of the plants (4/26) carried four to five copies of the transgene.

### Inheritance of Transgenes

The selfed and backcrossed progeny were evaluated for resistance to paromomycin and GUS expression in the T<sub>1</sub> seeds or T<sub>1</sub> plants. The segregation patterns of 50 events are shown in Table IV. Paromomycin-resistant and -sensitive seedlings or GUS-positive and -negative seeds or plants were clearly distinguishable by spraying the paromomycin on the seedlings or by histochemical GUS assay. A segregation ratio of 3:1 was observed for 22 out of 50 (44%) independent events, indicating a single functional GUS or NPT II gene locus. Twenty-two percent of the events (11/50) had two or more functional loci. Thirty-two percent (16/50) of the events had a non-Mendelian segregation pattern; that is the GUS or NPT II gene segregated at a 1:1 ratio, or the number of GUS-negative or paromomycin-sensitive plants was greater than the number of GUS-positive and paromomycin-resistant plants.

Six out of eight events (nos. 5, 15, 16, 17, 25, and 28) containing a single copy of the GUS gene based on Southern analysis showed a 3:1 segregation ratio of GUS-positive plants to GUS-negative T<sub>1</sub> plants. If more than one copy of



**Figure 3.** Southern analysis of T<sub>0</sub> transgenic events. DNA samples (15 µg) from 26 T<sub>0</sub> transgenic events (lanes 2–19 and 21–28; the lane number is the same as the event number) and one nontransformed wheat plant (lane 1) were digested with *Eco*RI, and the resulting fragments were resolved by electrophoresis and transferred to a membrane. The membrane was hybridized with a <sup>32</sup>P-labeled DNA probe corresponding to E35S promoter and the 5' intron of maize HSP 70 gene. In lane 20, 5 ng of pMON18365 DNA digested with *Eco*RI was loaded as a positive control. The positions and lengths, in kilobars, of the molecular size markers are indicated.

Mendelian segregation for paromomycin resistance and GUS expression was also observed in the T<sub>2</sub> progeny plants 28 and 49 as an example. Two-thirds of the GUS-positive T<sub>1</sub>

**Table IV.** Segregation of the *NPT II* and *GUS* genes in the  $T_1$  progeny

Events	T <sub>1</sub> Plants Assayed by Paromomycin Spray			T <sub>1</sub> Plants Assayed for GUS Activity		
	Resistant (R)	Sensitive (S)	R/S	Positive (+)	Negative (-)	+/-
3	14	17	1:1	14	7	1:1
4	20	11	3:1	20	11	3:1
5	24	11	3:1	24	11	3:1
6	28	6	3:1	28	6	3:1
7	33	1	15:1	33	1	15:1
8	29	6	3:1	29	6	3:1
9	26	6	3:1	26	6	3:1
10	14	17	1:1	14	17	1:1
11	11	23	1:2	11	23	1:2
12	32	0	32:0	32	0	32:0
13	29	7	3:1	29	7	3:1
14	12	22	1:2	12	22	1:2
15	30	5	3:1	30	5	3:1
16	21	9	3:1	21	9	3:1
17	32	4	3:1	32	4	3:1
18	52	9	3:1	59	1	63:1
19				78	17	3:1
21	30	8	3:1	0	40	0:40
22				74	0	74:0
23				28	32	1:1
25				74	27	3:1
26				2	98	1:49
28	24	9	3:1	24	9	3:1
29	37	1	15:1	37	1	15:1
30	34	3	15:1	34	3	15:1
31	35	0	15:0	35	0	15:0
32	24	10	3:1	24	10	3:1
33	32	2	15:1	32	2	15:1
34	26	6	3:1	26	6	3:1
35	27	8	3:1	27	8	3:1
36	3	30	1:10	3	30	1:10
37	6	21	1:3	6	21	1:3
38	8	13	1:2	8	13	1:2
39	1	34	1:34	1	34	1:34
41	8	15	1:1	8	15	1:1
42	28	6	3:1	28	6	3:1
43	33	0	15:0	33	0	15:0
44	13	1	15:1	13	1	15:1
45	18	5	3:1	18	5	3:1
46	20	0	15:1	20	0	15:1
48	7	5	1:1	7	5	1:1
49	12	2	3:1	12	2	3:1
50	6	20	1:3	6	20	1:3
51	25	6	1:3	25	6	1:3
52	22	14	1:1	22	14	1:1
53	28	8	3:1	28	8	3:1
54	10	10	1:1	10	10	1:1
55	21	9	3:1	21	9	3:1
56				22	9	3:1
57	44	13	3:1	47	13	3:1

<sup>a</sup> NA, Not analyzed; ?, the functional loci could not determined based on the segregation data because of the non-Mendelian segregation fashion in those events.

the gene was inserted in the plant genome, the estimated functional loci based on the segregation data were less than the copy number measured by Southern analysis in almost all of the cases (nos. 4, 6, 7, 8, 9, 13, and 19). The consistency of functional loci and the real copy number was observed in only one event (no. 18), which contained three functional loci and three copies of the gene.

The segregation ratios in the  $T_1$  progeny from the reciprocal crosses of  $T_0$  events 13, 28, 29, 30, and 60 are summarized in Table V. Events 13 and 28 had the 3:1 segregation ratio in the selfed progeny, whereas the progeny from the reciprocal crosses had a 1:1 segregation ratio. This result indicates that the transgenes were able to pass to the progeny through both male and female gametes.

The  $T_1$  progeny from  $T_0$  plants 18 and 28, which gave segregation patterns of 63:1 and 3:1 for GUS expression, respectively, were analyzed by Southern hybridization (Fig. 4). The  $T_0$  plant 18 had three inserts, and two of the  $T_1$  plants had exactly the same bands as their parent (lanes 3 and 4). Two other  $T_1$  plants (lanes 5 and 6) had a band with the same size, and another band with a distinct size, indicating that the DNA coding the GUS gene segregated in the progeny. These results suggest at least two independent inserts in event 18. Because  $T_0$  event 18 gave a segregation pattern of 63:1 for GUS expression, all three inserts should be independent. The  $T_0$  event 28 had one insert (lane 7), and two of the  $T_1$  plants (lanes 9 and 10) had the same

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**Table V.** Segregation of the *NPTII* gene in the progeny from the reciprocal crosses

Crosses	Resistant Plants (R)	Sensitive Plants (S)	R:S
no.			
No. 13 selfing	26	6	3:1
No. 13 X BW	8	3	1:1
BW X 13	20	13	1:1
No. 28 selfing	49	20	3:1
No. 28 X BW	11	15	1:1
BW X 28	5	8	1:1
No. 29 selfing	67	4	15:1
No. 29 X BW	9	2	3:1
BW X 29	10	4	3:1
No. 30 selfing	69	3	15:1
No. 30 X BW	22	9	3:1
BW X 30	16	4	3:1
No. 60 selfing	41	17	3:1
No. 60 X BW	16	12	1:1
BW selfing	0	76	NA

<sup>a</sup> BW, cv Bobwhite.

plants from both  $T_0$  plants produced GUS-positive and GUS-negative  $T_2$  at a ratio of 3:1. The  $T_2$  progeny of the remaining one-third were exclusively GUS positive. The  $T_2$  progeny of GUS-negative  $T_1$  plants from both plants maintained the same expression pattern. These results suggested that the  $T_1$  generation segregated into both homozygotic and heterozygotic plants, and that the transgenes were stably passed to their progeny in a Mendelian fashion.

## DISCUSSION

We are reporting a rapid transformation method for wheat via *A. tumefaciens*. Our results showed strong evidence that the T-DNA was stably integrated into the wheat genome and transmitted to the progeny. Over 100 independent transformants have been regenerated, and one-half of them were characterized. This study and the studies on rice and maize transformation mediated by *A. tumefaciens* (Hiei et al., 1994; Ishida et al., 1996) have provided strong support that monocotyledons can be transformed as dicotyledons using *A. tumefaciens* by manipulating various factors such as explant tissues, inoculation, and co-culture conditions, as well as the *A. tumefaciens* strain and the combination of the *A. tumefaciens* strain and plasmid.

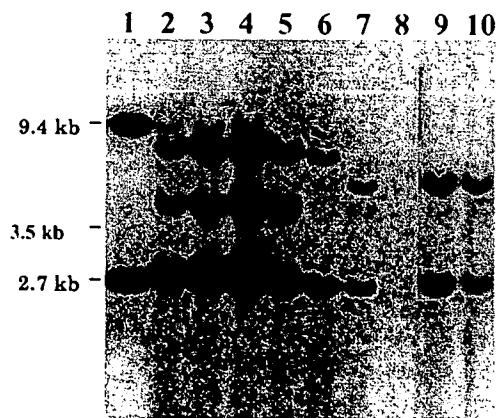
All of the studies of *A. tumefaciens*-mediated transformation of maize or rice used two strains, A281 or its derivative, EHA101 (Hood et al., 1986), and LBA4404 (Chan et al., 1993; Hiei et al., 1994; Aldemita and Hodges, 1996; Ishida et al., 1996; Rashid et al., 1996). The performance of the so-called "super-virulent" strain has been emphasized in some of the reports. Successful transformation of maize using *A. tumefaciens* was reported only when the "super-binary" vector was used. In the present study a nopaline *A. tumefaciens* strain C58 carrying the "ordinary" binary vector was used for infecting various explants, and this strain appeared to work efficiently. Stable transformants could be obtained from nonregenerable wheat suspension-cultured cells, and from regenerable immature embryos and embry-

ogenic calli. Transgenic plants were successfully produced from all of the regenerable explants used.

Various factors influenced the T-DNA delivery and stable transformation efficiency. Inoculation and co-culture conditions can be varied so as to favor the plant cell survival. Different tissues or cells exhibited various abilities to survive after *A. tumefaciens* infection. For example, precultured immature embryos, embryogenic calli, and suspension cells, which were cultured for a period of time prior to inoculation, showed better survival than the freshly isolated immature embryos. Therefore, higher *A. tumefaciens* cell densities, higher concentrations of the surfactant, and longer amounts of time may be used for inoculating these explants. Acetosyringone and Glc can be added to the inoculation and co-culture media, particularly when using the freshly isolated immature embryos.

The T-DNA delivery efficiency was significantly decreased when acetosyringone was absent. The similar observation was also noticed in rice and maize transformation (Hiei et al., 1994; Ishida et al. 1996). However, in our study with wheat suspension-cultured cells, exogenous induction agents such as acetosyringone and Glc were not necessary for the stable transformation (M. Cheng, J.E. Fry, C.M. Hironaka, and T.W. Conner, unpublished data). These results suggested that different tissues or cell types may have different competence for *A. tumefaciens* infection. Based on our results, the acetosyringone and Glc were recommended to be included in the inoculation and co-culture media for the stable transformation of the regenerable explants.

Surfactant present in the inoculation medium was one of the important factors noticed in this study. Two surfactants, Silwet and pluronic F68, proved to have a positive effect on the T-DNA delivery. The possible explanation for the effect the surfactants on enhancing T-DNA delivery might be the surface-tension-free cells favoring the *A. tumefaciens* attachment. We also tested other surfactants such



**Figure 4.** Southern analysis of  $T_1$  progeny from  $T_0$  plants 18 and 28. Southern blots were made as described in Figure 3. Lane 1, pMON18365 DNA as a positive control. Lane 2, DNA samples from  $T_0$  plant 18. Lanes 3 through 6, DNA samples from  $T_1$  progeny of  $T_0$  plant 18. Lane 7, DNA sample from  $T_0$  plant 28. Lane 8, DNA sample from GUS-negative  $T_1$  plant of  $T_0$  plant 28. Lanes 9 and 10, DNA samples from  $T_1$  plants of  $T_0$  plant 28. The positions and lengths, in kilobars, of the molecular size markers are indicated.

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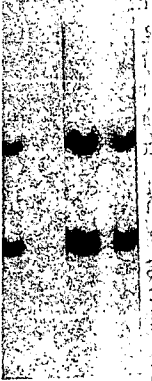
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as Tween 20 and Triton X, which appeared to be too toxic to the wheat tissues even when only a small amount was added to the inoculation medium.

This transformation system was efficient and required only 2.5 to 3 months from inoculation to transfer of the plants to soil. The transformation efficiency was as high as 4%. Most of the published studies on wheat transformation by the biolistic method showed that it took a fairly long time for tissue culture and regeneration (from 12–28 weeks) (Vasil et al., 1993; Weeks et al., 1993; Becker et al., 1994; Nehra et al., 1994; Zhou et al., 1995; Ortiz, et al., 1996), and the transformation efficiency was from 0.1 to 5.7%. Altpeter et al. (1996) reported a protocol for accelerated production of transgenic wheat by particle bombardment in which 8 to 9 weeks were required to produce transgenic plants after the initiation of culture and the transformation efficiency was up to 2%. Using the same regeneration and selection protocol presented in this paper, up to 20% transformation efficiency can be achieved in our laboratory through the biolistic approach. We think that once the inoculation and co-culture conditions are further optimized to obtain efficient T-DNA delivery with conditions favoring plant cell recovery, the transformation efficiency may be improved to as high as with the biolistic method.

Southern analysis showed different hybridization patterns among all of the tested T<sub>0</sub> transformants, indicating that T-DNAs were randomly integrated into the wheat genome. The T-DNA fragments that hybridized to the probe consisting of the E35S promoter and the maize HSP70 intron clearly did not derive from the vectors in the free *A. tumefaciens* cells that might exist in the plants regenerated from inoculated explants; otherwise, there would have been two bands, as in the control lane. Based on the samples tested, approximately 35% of the plants have single inserts, which was close to that observed in rice (32%) (Hiei et al., 1994), but significantly lower than that in maize (60–70%) (Ishida et al., 1996). The differences could be due to the plant species, explant types, or other factors such as *A. tumefaciens* strain and plasmid. The number of events with a single insert produced using the *A. tumefaciens*-mediated transformation was significantly higher than that with the biolistic method. Using similar constructs, the same cultivar and regeneration and selection protocol, 77 events were produced via the biolistic method in our laboratory. Only 17% (13/77) plants contained single copies of transgenes (data not shown).

The genetic analysis of T<sub>1</sub> and T<sub>2</sub> progeny also provided strong evidence of the incorporation of T-DNA into the wheat genome. The NPT II and GUS genes were inherited to the T<sub>1</sub> and T<sub>2</sub> generations in a Mendelian fashion in most of the events. The data from the Southern analysis of the T<sub>1</sub> generation supported the genetic data in most of the cases, although non-Mendelian segregation patterns were observed occasionally. Similar results were also reported in rice and maize (Hiei et al., 1994; Ishida et al., 1996) and in dicot species transformed by *A. tumefaciens* (Hobbs et al., 1990; Ulian et al., 1994). Gene silence and nondetectable gene expression level in the transgenic plants might be partially responsible for causing the abnormal segregation patterns.

In summary, we have developed a method for rapid production of transgenic plants via *A. tumefaciens* from three kinds of explants of wheat. The transformation efficiency was 0.14 to 4.3% based on the experiments that produced the transgenic plants. The transformed plants appeared to be morphologically identical to nontransformed, growth chamber-grown control plants. In most of the cases, the transformed genes behaved as dominant loci exhibiting normal Mendelian segregation. Therefore, an *A. tumefaciens*-mediated transformation system is now available as an alternative routine method for genetic transformation of wheat.

ACKNOWLEDGMENTS

The authors are grateful to Dr. C.L. Armstrong for critical review of the manuscript. Special thanks also go to Drs. M.A.W. Hinchee, G. Ye, and J.R. Rout for their support, to A.F. Schmitt and W.J. Feldker for their assistance in making culture media, and to B.D. Rhodes for providing secretarial assistance.

Received May 16, 1997; accepted August 15, 1997.  
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## **EXHIBIT D**

## A new, endosperm-supported callus induction method for wheat (*Triticum aestivum* L.)

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Received 15 May 1989; accepted in revised form 8 January 1990

**Key words:** callus culture, mature embryo, mesocotyl, organogenesis, *Triticum aestivum*

### Abstract

A new, endosperm-supported callus induction method was developed using mesocotyls of mature wheat embryos. After seed germination under aseptic condition, most of the germ tissues were cut off and only a few mm of the mesocotyl tissue with the scutellum was used for callus induction. The seeds were placed furrow downwards in 2,4-D solution ( $6\text{--}8\text{ mg l}^{-1}$ ). Proliferating callus tissues were already observed on the cut surface of the mesocotyls on the 2nd day after inoculation. On the MS nutrient medium, callus formation from the isolated scutella with attached mesocotyls was negligible even after 6 days. For shoot and root regeneration, the calli produced up to 10 days were removed from the seeds and transferred onto a hormone-free MS medium. As shown by histological methods, the plantlets regenerated via organogenesis.

### Introduction

The application of somatic tissue cultures in plant sciences requires efficient callus initiation and plant regeneration techniques. Reproducible callus induction and plantlet regeneration have already been reported for a number of graminaceous species [7, 25, 26], a group of higher plants formerly regarded as difficult objects for in vitro manipulation.

In wheat, one of the most important monocotyledonous crop species, several explants have been used for somatic callus culture: immature embryos [15, 17, 22, 23], immature leaves [2, 29], immature inflorescences [4, 6, 8, 10, 16, 20], mature embryos [3, 13, 21], mesocotyls [27, 28], seeds [10] and apical meristems [18]. Currently, the immature embryos and inflorescences are the most frequently used explant sources for the initiation of wheat callus cultures. Nevertheless, these explants are available in the field only within limited periods of the year, or the donor plants have to be grown in growth chambers or greenhouses with extra effort. Further,

callus induction on culture media takes place after a rather long lag-phase, which is necessary for the stress adaptation of the explants.

In this article, we describe a method in which only mature seeds and 2,4-D are required for callus induction in wheat. The induction process is very rapid: instead of synthetic compounds, the endogenous metabolites formed enzymatically in the germinating endosperm serve as nutrients for the responsive cells of the mesocotyl tissues. A less rapid and more tedious mesocotyl-based system for the establishment of callus cultures and regeneration in barley was developed by Jelaska et al. [12], using mesocotyls from 7-day-old seedlings, grown on a nutrient medium supplemented with 2,4-D or 2,4,5-T.

### Materials and methods

Mature seeds of *Triticum aestivum* L. cv. GK Kincső were used.

The seeds were surface-sterilised for 4 min in 90% v/v ethanol and rinsed three times with sterile

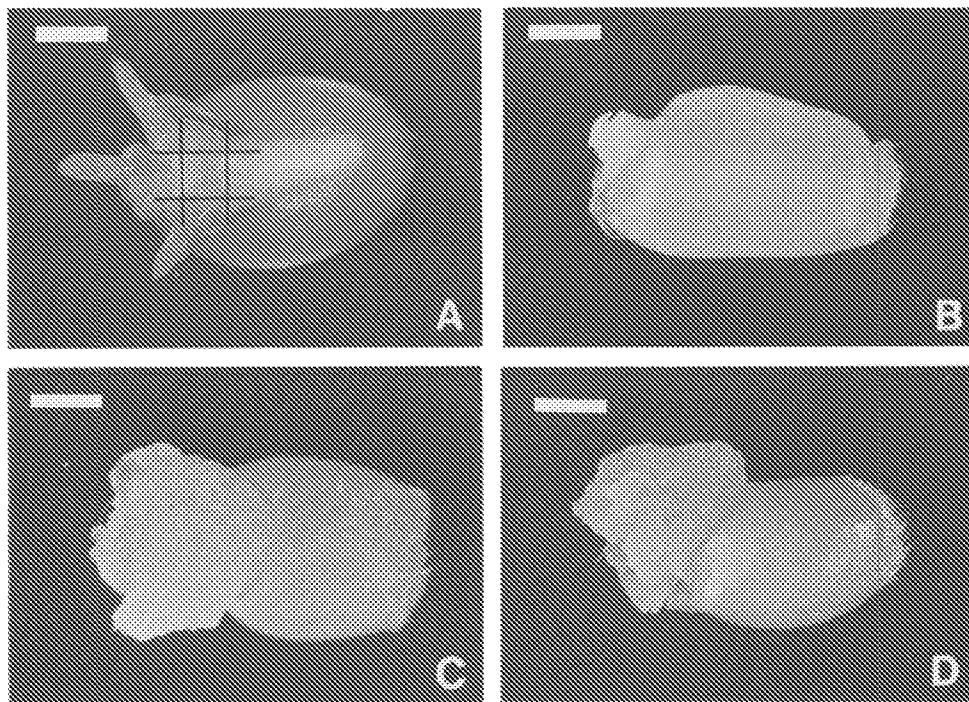


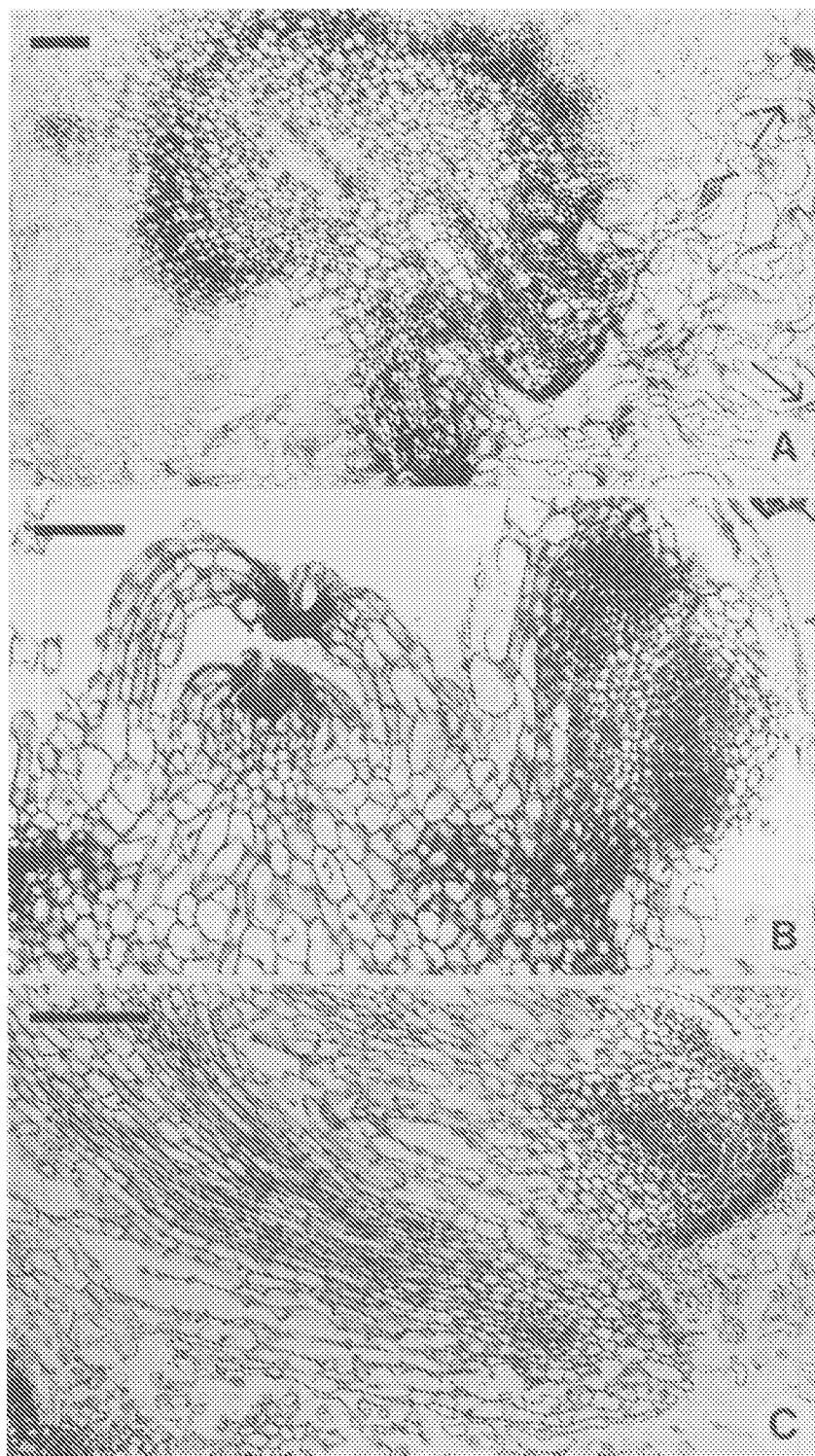
Fig. 1. Mesocotyl explant preparation and callus formation in the endosperm-supported wheat callus induction system. Germinating seed with indicated cutting lines (A), the prepared embryo with mesocotyl attached to the seed (B), 4-day-old calli formed on the seed (C) view from above, (D) lateral view. Bar = 2 mm.

deionised water. The seeds were then imbibed in sterile water at 28°C for 5 hrs and sterilised again for 25 min in 50% v/v commercial bleach containing a few drops of Tween 80. Finally, the seeds were rinsed with six changes of sterile deionised water. Germination was performed on sterile filter paper in 100 × 15 mm Petri dishes for 26 hrs at 28°C. Before callus induction, most of the germ tissues were removed by means of four cuts with a scalpel, so that only mesocotyl remained on the scutellum (Fig. 1A, B).

For callus induction, the seeds were placed furrow downwards in sterile 50 × 8 mm Petri dishes (three seeds per dish) containing 2 ml of 2,4-D solution (8 mg l<sup>-1</sup>). In parallel with this, separated scutella plus mesocotyls were put on MS medium supplemented with 2,4-D (8 mg l<sup>-1</sup>) and cultured as the seeds with scutella and mesocotyls. The Petri dishes were incubated at 26°C in the dark for 9 days. At this time, the developed calli were removed from the seeds and transferred onto a hormone-free medium in 100 × 15 mm Petri dishes

(ten calli per dish). The culture medium consisted of Murashige and Skoog's [19] mineral salts, glycine (2 mg l<sup>-1</sup>), pyridoxine.HCl (0.5 mg l<sup>-1</sup>), nicotinic acid (0.5 mg l<sup>-1</sup>), sucrose (20 g l<sup>-1</sup>) and Oxoid agar (7 g l<sup>-1</sup>). The pH of the 2,4-D solution and culture media was adjusted to 5.8 with NaOH or HCl before the addition of agar, and they were autoclaved at 120°C for 15 min.

The transferred callus cultures were incubated at 25°C in the dark for 3 weeks. Thereafter, they were grown at 23°C in a 16 hr/8 hr light-dark cycle provided by 40 W Tungsram F33 white fluorescent tubes (2000 lux). When green spots appeared on the surface, some of the calli were fixed for histological studies in ethanol 50% v/v:glacial acetic acid:formalin (90:5:5) solution, dehydrated through ethanol:xylene series and embedded in paraffin containing 5% honey wax. Serial sections of 10 µm were cut with a Leitz 1208 slicing microtome and stained with 2% w/v toluidine blue. The microscopic slides with the sections were covered with DePex (Serva) and photographed.



*Fig. 2. Organogenesis from cultured mesocotyls of mature wheat embryos. De novo formation of meristematic tissue (A), an adventitious shoot bud (B), and differentiation of roots (C). Note the elongated, tubular cells on A (arrows). Bar = 160  $\mu$ m.*



Fig. 3. Comparison of proliferation of wheat mesocotyls attached to the seed in 2,4-D solution (lower row) and of separate mesocotyls plus scutella on 2,4-D-containing MS nutrient medium (upper row). Culture age: 4 days each. Bar = 4 mm.

### Results and discussion

In the callus induction system described above, callus initiation occurred on the 2nd day after inoculation. On the 4th day, the average diameter of the calli was 4 mm (Fig. 1C, D), and 9-day-old calli were ready for subculture. With the described culture system, all mesocotyls produced callus. Callus induction with barley mesocotyl explants was 75–100% in the experiments of Jelaska et al. [12], but the time needed for callus production was not indicated.

The mesocotyl-derived calli were friable and translucent (Fig. 1C, D), consisting of loosely associated, elongated tubular cells (Fig. 2A). A relatively high 2,4-D concentration ( $6\text{--}8\text{ mg l}^{-1}$ ) was a prerequisite of good callus formation. This observation is in agreement with that of Jelaska et al. [12]. At lower 2,4-D concentrations ( $2\text{--}4\text{ mg l}^{-1}$ ), direct shoot differentiation was observed in some cases.

For various cereal explants, the time necessary for visible callus development has been found to be 10–21 days [1, 8, 9, 16, 17, 24, 26]. However, the present technique furnishes wheat calli suitable for subculture within 10 days. This is probably due to a concerted effect of wounding, meristematic activity in the mesocotyl and a natural, perhaps more favourable nutrient medium, the enzymatically

hydrolysed endosperm being in intimate, undisturbed contact with the scutellum. In this way, the callus-forming mesocotyl does not need to adapt to the synthetic inducing medium. The results are clearly demonstrated by Fig. 3: large calli developed on the endosperms after 4 days, while the excised mesocotyl + scutellum explants became swollen at most, but did not proliferate when cultured on MS medium for the same time.

It is a generally accepted view that mature embryo-derived cereal tissue cultures are of limited practical significance, because of their low regenerating capacity [5, 11, 13, 14]. On hormone-free MS medium in the light, green spots appeared on our calli in the 5th week of subculture. Serial sections prepared from such callus tissues revealed *de novo* meristem differentiation, and adventitious shoot bud and root formation (Fig. 2A–C). Later, these organogenic calli regenerated complete green plantlets.

Although our quantitative studies on this point are not yet completed, the mature embryo-derived calli produced by the endosperm-supported system seem to be suitable for the regeneration of complete wheat plants in one step at an acceptable rate. By means of the endosperm-supported callus induction method, the total time of callus production and plant regeneration can be shortened. This is an important aspect as concerns breeding application.

Besides its rapidity and simplicity, a further advantage of this method is that it starts with mature seeds, which are available without limit at any time.

Among other questions, the behaviour of additional wheat varieties and other cereal species will be studied in this system.

### Acknowledgements

The authors express their thanks to Éva V. Bogdán for her skilful technical assistance, to Dénes Dudits for his advice, to Gábor Takács for the photographs, and to David Durham for revision of the English text.

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## **EXHIBIT E**

## Rapid Production of Multiple Independent Lines of Fertile Transgenic Wheat (*Triticum aestivum*)<sup>1</sup>

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Improvement of wheat (*Triticum aestivum*) by biotechnological approaches is currently limited by a lack of efficient and reliable transformation methodology. In this report, we detail a protocol for transformation of a highly embryogenic wheat cultivar, Bobwhite. Calli derived from immature embryos, 0.5 to 1 mm long, were bombarded with microprojectiles coated with DNA containing as marker genes the *bar* gene, encoding phosphinothricin-resistance, and the gene encoding  $\beta$ -glucuronidase (GUS), each under control of a maize ubiquitin promoter. The bombardment was performed 5 d after embryo excision, just after initiation of callus proliferation. The ability of plantlets to root in the presence of 1 or 3 mg/L of bialaphos was the most reliable selection criteria used to identify transformed plants. Stable transformation was confirmed by marker gene expression assays and the presence of the *bar* sequences in high molecular weight chromosomal DNA of the resultant plants. Nine independent lines of fertile transgenic wheat plants have been obtained thus far, at a frequency of 1 to 2 per 1000 embryos bombarded. On average, 168 d elapsed between embryo excision for bombardment and anthesis of the T<sub>0</sub> plants. The transmission of both the resistance phenotype and *bar* DNA to the T<sub>1</sub> generation verified that germline transformation had occurred.

Many of the recent advances in plant science have resulted from application of the analytical power of recombinant DNA technology coupled with plant transformation. These approaches facilitate studies of the effects of specific gene alterations on plant development and physiology. They also make possible the direct manipulation of genes to bioengineer improved plant varieties.

Monocotyledonous plants, and cereal crops in particular, have lagged behind dicotyledonous plants in ease and efficiency of transformation. Rice was the first major cereal crop transformed. Toriyama et al. (1988), Zhang and Wu (1988), and Shimamoto et al. (1989) all used direct DNA delivery into regeneration-competent protoplasts to obtain transgenic plants. Although this approach continues to be the main procedure for rice transformation (Peng et al., 1992), an alternative was developed by Christou et al. (1991), who used immature embryos as the target tissue and electric discharge particle acceleration as the DNA delivery method.

Because it is difficult to regenerate fertile plants from

protoplasts of cereals other than rice, the initial production of fertile transgenic maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), oat (Somers et al., 1992), and sugarcane (Bower and Birch, 1992) plants used embryogenic suspension cell or callus cultures as target tissues and microprojectile bombardment as the mechanism of DNA delivery. In all these cases, the ability to regenerate plants depended on the establishment of long-term embryogenic cell cultures. Recently, it has been shown that the establishment of such cultures is not necessary for successful transformation of maize: D'Halluin et al. (1992) used wounded immature embryos as target tissues and electroporation for DNA delivery to produce transformed plants.

Although wheat (*Triticum aestivum*) is the largest crop in the world in terms of production, it was the last among economically important cereals to be transformed. Vasil et al. (1991) produced stably transformed wheat suspension-cell cultures from which they were unable to regenerate plants. More recently, Vasil et al. (1992) obtained several transformed callus lines after microprojectile bombardment of embryogenic callus and selection with the herbicide Basta. From one of these lines, transformed wheat plants were regenerated. These plants were unable to self-fertilize, but progeny could be produced by outcrossing to either wild-type pollen or ova. These T<sub>1</sub> progeny were fully fertile and transmitted the transformed phenotype to at least the T<sub>2</sub> generation.

Although the work of Vasil and collaborators is a landmark in efforts to develop wheat transformation, the protocol they report is limited in its utility by its dependence on the identification and establishment of a specific callus type in long-term tissue cultures. Establishment of the regenerability of such cultures requires several months, is limited to certain genotypes, and declines with time (Redway et al., 1990; Vasil et al., 1992). To circumvent these limitations, we reasoned that a more suitable target for wheat transformation might be the callus produced by immature embryos shortly after excision and culturing. Immature embryos of the hexaploid wheat cultivar Bobwhite form such callus tissue, which can be maintained and regenerated into fertile plants with high

<sup>1</sup> Supported by Agricultural Research Service No. 5325-21430-007-00D.

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Abbreviations: *bar*, the sequences of the *bar* gene from *Streptomyces hygroscopicus* that encode phosphinothricin acetyl transferase and confer resistance to bialaphos and Basta; GUS,  $\beta$ -glucuronidase, encoded by the *uidA* gene from *Escherichia coli*; MS medium, Murashige and Skoog medium; PAT, phosphinothricin acetyl transferase.

frequency (T. Weeks, unpublished data; J. Driver, A. Guenzi, and T. Peeper, unpublished data). We now report use of this tissue as the target for DNA delivery by microprojectile bombardment and a protocol for wheat transformation that has resulted in the production of multiple independent lines of fertile transgenic plants.

## MATERIALS AND METHODS

### Callus Culture and Plant Growth Conditions

Wheat plants (*Triticum aestivum* L. em. Thell. cv Bobwhite) were grown in a greenhouse in Albany, CA. To establish callus cultures, caryopses 10 to 18 d postanthesis were surface-sterilized with 70% ethanol for 5 min and 20% sodium hypochlorite for 15 min, followed by two changes of sterile distilled water. Immature embryos, 0.5 to 1 mm long, were aseptically removed under a stereo dissecting microscope and placed with the scutella exposed on MS medium (Murashige and Skoog, 1962) modified for wheat cell culture (Sears and Deckard, 1982) and solidified with 0.25% (w/v) Phytigel (Sigma Chemical Co.<sup>2</sup>). Calli cultures were maintained at 27°C with a 16-h photoperiod (43  $\mu\text{E}/\text{m}^2$ ) on MS medium with 2% Suc and 1.5 mg/L of 2,4-D and transferred to new medium at 2-week intervals. For regeneration, embryogenic calli were transferred to MS medium with 0.5 mg/L dicamba (Sandoz Crop Protection, Des Plaines, IL) as described by Hunsinger and Schaub (1987). Calli-derived shoots were transferred to Pyrex culture test tubes (25  $\times$  150 mm) containing rooting media composed of half-strength MS without hormones. For selection after bombardment, agar media at each stage were supplemented with 1 mg/L of bialaphos (Meiji Seika Kasha, Tokyo, Japan).

Plantlets were transferred from rooting media to pots of Sunshine soil mixture No. 1 (Fisons Horticulture Co., Mississauga, Canada) and acclimated to lower humidity at 21°C with a 16-h photoperiod (300  $\mu\text{E}/\text{m}^2$ ) in an environmental chamber. After 2 weeks, plants were transferred to the greenhouse. These primary regenerants are called  $T_0$  plants. The first generation progeny of these plants are called  $T_1$  plants. These were obtained by excising embryos from  $T_0$  plants 15 DAF and germinating them on MS medium solidified with 0.25% Phytigel agar.

### Plasmid DNA

Plasmid pAHC25, the vector used for wheat transformation, was kindly provided by Alan Christensen and Peter Quail (Plant Gene Expression Center, University of California Berkeley/U.S. Department of Agriculture, Albany, CA). This dual-expression vector consists of the *uidA* (Jefferson et al., 1987) and *bar* (Thompson et al., 1987) genes, each under control of the maize ubiquitin *Ubi1* promoter (Christensen et al., 1992; A.H. Christensen and P.H. Quail, unpublished data). The *bar* gene encodes the enzyme PAT, which inactivates phosphinothricin, the active ingredient of the herbicides bialaphos and Basta. The *uidA* gene encodes the enzyme

GUS. Plasmid DNA was purified from alkaline-lysed cells on CsCl gradients and stored at a concentration of 1 mg/mL in Tris-EDTA buffer, pH 8.0 (Sambrook et al., 1989).

### Microprojectile Bombardment

Prior to bombardment, 1- $\mu\text{m}$  gold particles were coated with pAHC25 DNA by the procedure of Daines (1990). A stock suspension of gold particles (Bio-Rad) was suspended at 60 mg/mL in absolute ethanol. Thirty-five microliters of the suspension were aliquoted into 1.5-mL microcentrifuge tubes, washed in sterile distilled water, and resuspended in 25  $\mu\text{L}$  of Tris-EDTA containing 25  $\mu\text{g}$  of supercoiled plasmid DNA. The following solutions were added in order: 220  $\mu\text{L}$  of sterile water, 250  $\mu\text{L}$  of 2.5 M  $\text{CaCl}_2$ , and 50  $\mu\text{L}$  of 0.1 M spermidine (free base). The microcentrifuge tubes were shaken with a vortex mixer at 4°C for 10 min and centrifuged at 16,000g for 5 min. The supernatant was removed, and the pellet was washed with 600  $\mu\text{L}$  of ethanol. The DNA-coated gold pellets were resuspended in 36  $\mu\text{L}$  of ethanol. For bombardment, 10  $\mu\text{L}$  of the DNA-gold suspension was placed in the center of a macroprojectile.

Approximately 25 embryos were placed in the center of a 15  $\times$  100 mm Petri dish containing callus maintenance medium solidified with 0.35% Phytigel. After 5 d in culture, the embryo-derived calli were bombarded under vacuum with pAHC25-coated gold particles, using the helium-driven DuPont Biolistic Delivery System (model PDS-1000) and disposable components supplied by Bio-Rad. The distance from the stopping plate to the target was 13 cm, and the rupture disc strength was 1100 p.s.i. Immediately after bombardment, calli were transferred to MS selection media containing 1 mg/L of bialaphos.

### Enzyme Assays

GUS activity (Jefferson et al., 1987) was assessed histochemically using the same buffer used by Perl et al. (1992) except that the substrate 5-bromo-4-chloro-3-indoyl- $\beta$ -D-glucuronic acid was purchased from Jersey Lab and Glove Supply (Livingston, NJ). PAT activity was determined in extracts prepared from 8-cm sections of leaf tips 1 week after regenerated plants were transferred to soil. The acetylation assay was performed as described by Spencer et al. (1990) except that a Wheaton hand grinder was used to prepare the extracts instead of a bead beater, and 2.5  $\mu\text{L}$  instead of 3  $\mu\text{L}$  of [ $^{14}\text{C}$ ]acetyl-CoA (55.9 mCi/mmol, New England Nuclear-Dupont) were used as label. The products of the reaction corresponding to 2.4  $\mu\text{g}$  of total protein were loaded into each lane of a Whatman (Maidstone, England) LHP-KDF high-performance TLC plate.

### Wheat DNA Isolation and Gel Blot Analysis

Wheat genomic DNA was isolated as described by D'Ovidio et al. (1992) and quantitated by measuring  $A_{260}$ . Fifty micrograms of each DNA sample were digested with *Hind*III in 300  $\mu\text{L}$  of the manufacturer's (GIBCO BRL) buffer overnight. Twenty-five micrograms of digested or undigested DNA were separated by electrophoresis through 0.6% aga-

<sup>2</sup> The use of a brand name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

rose (FMC Corp., Rockland, ME) gels in Tris-borate-EDTA (Sambrook et al., 1989) buffer. To reconstruct a single copy of plasmid per wheat hexaploid genome, 25  $\mu$ g of *Hind*III-digested nontransformed wheat DNA was mixed with 1.6  $\mu$ g of a 3.8-kb *Pvu*I/*Xho*I fragment of pAHC25 that includes the *bar* coding region.

For blot analysis, DNA was transferred (Southern, 1975) to a Nytran membrane (Schleicher & Schuell). DNA was fixed to the membrane by UV cross-linking, using a Stratalinker (Stratagene). Prehybridizations were carried out at 65°C for 3 h in a variant of the solution used by Devey et al. (1991): 4× SSPE (150 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 1 mM EDTA, pH 7.7), 10× Denhardt's reagent, 1% (w/v) SDS, 1% (w/v) nonfat dry milk, and 100  $\mu$ g/mL of denatured salmon sperm DNA. Hybridizations were carried out for 40 h at 65°C in 4× SSPE, 5× Denhardt's solution, 0.5% (w/v) SDS, 1% (w/v) nonfat dry milk, 50  $\mu$ g/mL of denatured salmon sperm DNA, 10% (w/v) dextran sulfate, and 50 × 10<sup>6</sup> cpm of <sup>32</sup>P-radiolabeled probe. The probe consisted of the *bar* coding region isolated as a 570-bp *Pst*I fragment from pAHC25 and labeled with [<sup>32</sup>P]dCTP (New England Nuclear) using the Random Primed DNA Labeling Kit supplied by Boehringer Mannheim Biochemicals. After hybridization, membranes were rinsed with 1× SSPE, 0.2% SDS at room temperature, and washed twice for 45 min each at 65°C in 0.25× SSPE, 0.2% SDS. Damp filters were exposed at -80°C on Kodak XAR-5 film with an intensifying screen.

#### Herbicide Application

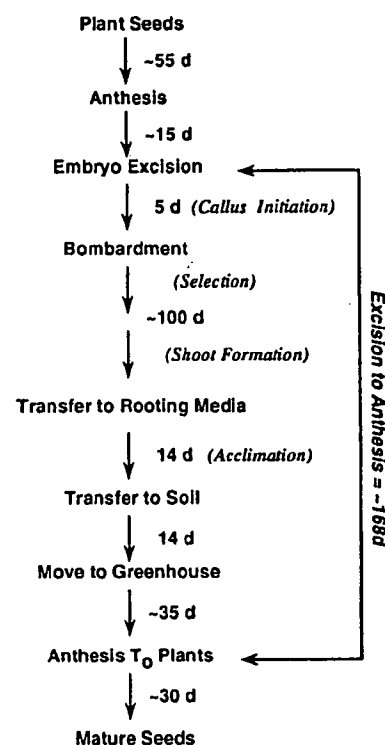
Basta TX (Hoechst AG, Frankfurt am Mein, Germany) is a commercial formulation of phosphinothricin (200 g/L). A 2% solution of the herbicide was sprayed on greenhouse-grown plants, which were then observed over a 3-week period.

### RESULTS

#### Initiation, Bombardment, and Selection of Callus Tissue

The wheat cv Bobwhite was chosen for use in transformation experiments because of its high frequency of regeneration from tissue culture to fertile plants (J. Driver, A. Guenzi, and T. Peeper, unpublished data). The steps of the transformation process are shown in Figure 1. Immature embryos 0.5 to 1 mm in length were excised from greenhouse-grown plants (10–18 d after anthesis, depending on the time of year) and placed, scutellum side exposed, on callus maintenance media containing 1.5 mg/L of 2,4-D (Fig. 2A). Five days after initiation into tissue culture, proliferating callus tissue is visible at the edges of the embryos (Fig. 2B). At this stage, the embryos were bombarded with gold particles coated with 7  $\mu$ g of the plasmid pAHC25, which contains the *bar* and GUS marker genes, each under control of a maize ubiquitin promoter (Christensen et al., 1992). The first experiment utilized older callus tissue and transient GUS expression to establish efficient DNA bombardment conditions. Figure 2C shows the pattern of ubiquitin:GUS gene expression in a piece of 30-d-old embryogenic callus visualized with the histochemical assay 2 d after bombardment. Discrete, intensely blue sectors are seen distributed over the entire surface. In the center of the target tissue, the staining appears confluent.

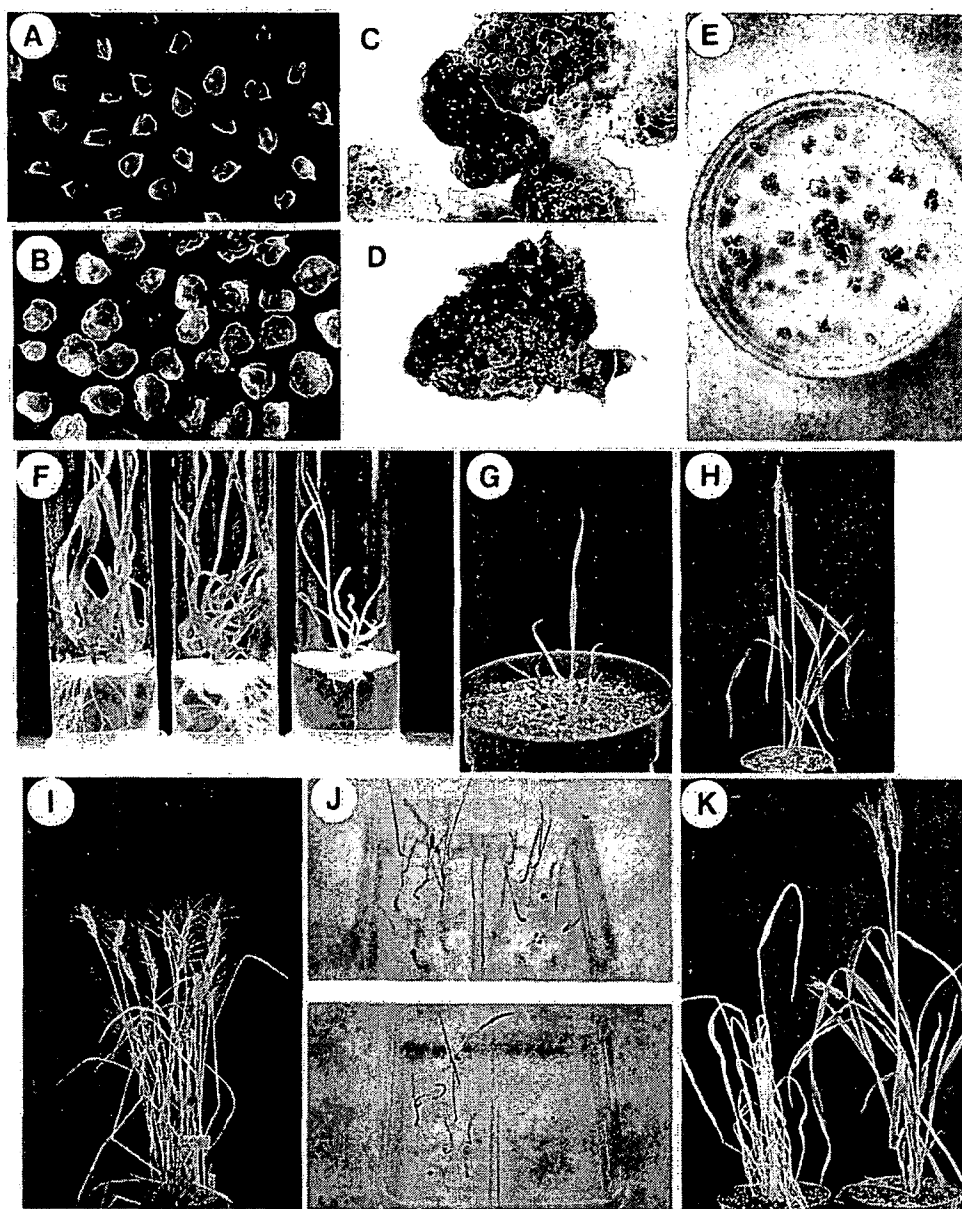
#### TIME FRAME FOR TRANSFORMATION OF CULTIVAR BOBWHITE



**Figure 1.** Time frame for events and processes in the production of transgenic Bobwhite plants. The times shown are averages for experiments performed in 1992. The times designated by ~ are approximate and vary with either the season of the year in which the plants are grown or the individual callus line or plant.

In subsequent transformation experiments, these bombardment conditions were used to deliver pAHC25 DNA to embryos 5 d after excision and culturing. Immediately after bombardment, the embryos were transferred to callus maintenance media containing 1 mg/L of bialaphos for selection of resistant tissues. In preliminary experiments, this level of bialaphos had inhibited growth of normal callus 27% in 3 weeks and 67% in 6 weeks (data not shown). Healthy, growing sectors of callus were transferred every other week to fresh selection media. By 6 to 7 weeks, resistant callus tissue could be clearly identified, and individual cell lines were established. These lines are designated in this report by their bombardment number followed by an alphabetical letter. When callus pieces from some of the lines were tested for GUS gene expression by the histochemical assay, the resistant callus displayed uniformly dark-blue staining (Fig. 2D), indicative of the presence and expression of the ubiquitin:GUS portion of the vector. Control callus remained yellowish-white in the presence of the substrate (not shown).

Resistant callus lines were maintained on selection media. At variable times after bombardment, green sectors, the precursors of shoots, originated on portions of the callus (Fig. 2E). At this stage, callus pieces were divided and the greening portions were transferred to regeneration media (MS with



**Figure 2.** Wheat callus and plant tissues. A, Immature Bobwhite embryos excised 15 d postanthesis on agar medium. B, Proliferation of callus tissue from embryos after 5 d on MS medium. C, Wheat callus tissue 30 d after culturing from immature embryos, stained histochemically for GUS activity 2 d after bombardment with pAHC25. D, Bialaphos-resistant tissue undergoing shoot (protrusion) regeneration, stained histochemically for GUS activity. E, Callus tissue on MS media containing 1 mg/L of bialaphos 70 d after bombardment. F, Regeneration of roots; Left, Nontransformed plantlet in rooting medium lacking bialaphos; center, putative transformant in rooting medium supplemented with 3 mg/L of bialaphos; right, nontransformed plantlet in rooting medium with 3 mg/L of bialaphos. G, Transformed plant from line 12M shortly after transfer to the greenhouse. H, Transformed plant from line 12K at the heading stage (grown during the winter months). I, Transformed plant from line 9C at maturity (grown during the summer months). J, Selection of  $T_1$  progeny on germination media. Immature embryos from a  $T_0$  plant of line 2A (left of each panel) and a nontransformed control plant (right of each panel) excised 15 DAF and germinated on MS-agar media with (lower) and without (upper) 3 mg/L of bialaphos. Seedlings were photographed after 18 d. K, Selection of  $T_1$  progeny at the boot stage. A,  $T_1$  plant from line 2A (right) and a nontransformed plant (left) photographed 2 weeks after spraying with 2% Basta.

0.5 mg/L of dicamba). In most cases, sufficient callus was present underlying the differentiating tissues to maintain the callus line on selection media. Usually, multiple plants could be regenerated from each line.

Differentiated shoots were transferred to culture tubes containing rooting media (half-strength MS) with 1 or 3 mg/L of bialaphos. Only about 10% of the shoots that had been able to organize on the regeneration media were able to form roots in media containing either level of bialaphos (Fig. 2F). The plantlets were scored as resistant at this stage by their ability to form long, highly branched roots in the bialaphos-containing medium (center in Fig. 2F), similar to the roots produced by nontransformed plantlets in medium lacking the herbicide (left in Fig. 2F). Sensitive plantlets (right in Fig. 2F) initiated root formation, but the primary root soon stopped growing and put out only a few short lateral roots. Aerial portions of sensitive plantlets exhibited yellow necrosis and reduced vigor within 1 week, whereas resistant plantlets thrived in the rooting media.

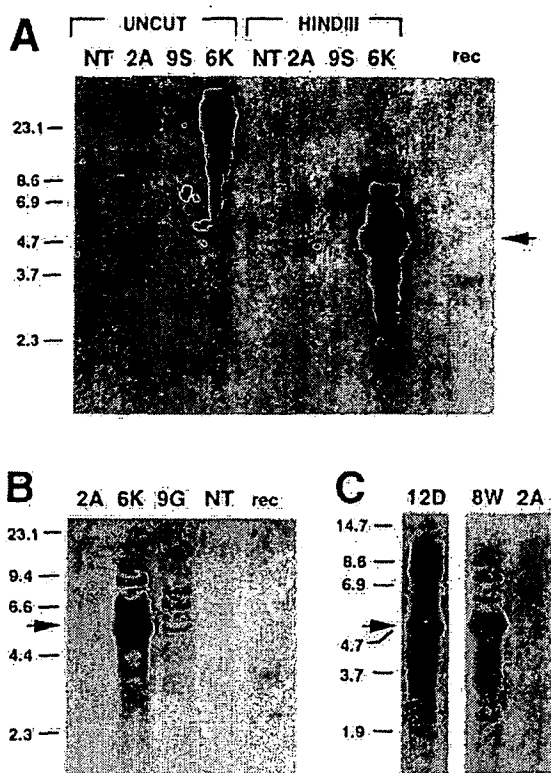
After 2 weeks, plantlets established under bialaphos selection were transferred to soil. In a growth chamber under high humidity, they were allowed to acclimate to greenhouse conditions for 2 weeks. Upon transfer to the greenhouse, the young plants exhibited the curled and spindly appearance (Fig. 2G) typical of nontransformed Bobwhite plants regenerated from tissue culture. As regenerated plants matured and flowered, they regained a normal appearance (Fig. 2, H and I). Some of the lines had normal levels of fertility and seed set. However, most had reduced seed set compared with nontransformed Bobwhite plants regenerated from tissue culture. Only one line, 6K, was completely sterile.

Figure 1 summarizes the transformation and regeneration process and shows average times required for each of the steps. Of the total of 168 d between excision of the target embryos and anthesis of regenerated plants, about 119 d of the process are spent in tissue culture. Exact times varied for each regenerant, depending on how quickly resistant callus lines were established and how quickly shoots developed.

#### DNA Analysis

DNA was extracted from leaf tissue of  $T_0$  plants derived from independent callus lines and analyzed for the presence of *bar* DNA (Fig. 3). DNA from nontransformed plants (lanes labeled NT) exhibit no hybridization to the *bar* coding region fragment used as probe. Lanes containing uncut DNA from plants 2A, 9S, and 6K show hybridization to a DNA band of high mol wt. This result is indicative of integration of *bar* sequences into wheat chromosomal DNA.

*Hind*III-digested DNAs from six independent transformation events are shown in panels A, B, and C of Figure 3. DNA from line 2A is included in each panel so that the relative intensities of the bands can be compared between the different blots. As expected for independent events, each plant had a unique integration pattern of DNA fragments. Two events obtained from bombardment 9, but selected as independent callus lines, have different patterns (lanes 9G and 9S). The patterns ranged from simple to complex. DNA from line 2A contains only two bands with homology to the *bar* probe, each present at about one copy per hexaploid

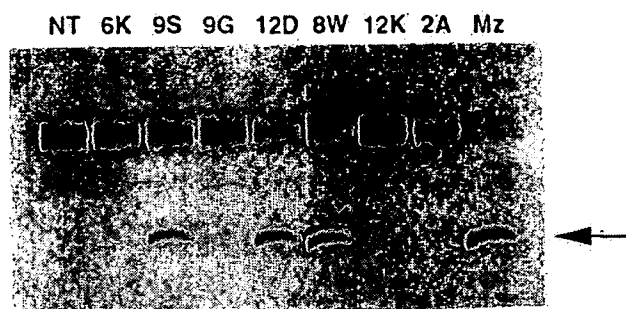


**Figure 3.** DNA gel-blot analysis of transformants. Each lane contains 25  $\mu$ g of total leaf DNA. Blots were hybridized with the coding region of the *bar* gene. Arrows mark the migration position of the 5.5-kb *Hind*III fragment of pAHC25 that is homologous to the *bar* probe. The migration position of markers are shown to the left of each panel with sizes in kb. A, Autoradiogram of DNA from transformed lines 2A, 9S, and 6K. The left four lanes contain undigested DNA; the middle four labeled lanes contain DNA digested with *Hind*III. The lanes marked NT contain DNA from nontransformed leaves. The lanes labeled "rec" contain 25  $\mu$ g of DNA from nontransformed leaves and 1.6  $\mu$ g of the ubiquitin:*bar* sequences of pAHC25 digested with *Pvu*I and *Xho*I (single copy reconstruction). B, Autoradiogram of *Hind*III-digested DNAs from transformed lines 2A, 6K, and 9G. C, Autoradiogram of *Hind*III-digested DNAs from transformed lines 12D, 8W, and 2A.

genome as judged by comparison of their intensities with that of the band in the single-copy reconstruction lane (rec). Neither of the bands is of the 5.5-kb size expected for the intact plasmid digested with *Hind*III (arrow), indicating that a rearrangement has occurred to alter the size of this fragment or that one or both of the flanking *Hind*III sites has been lost. Line 6K has the highest copy number of fragments homologous to *bar*, estimated to be about 35 on another gel by comparison with several reconstruction lanes (data not shown). In this and other lines with high copy numbers, most of the copies are the size of the intact plasmid cut with *Hind*III. In addition, these lines have many other sizes of fragments, indicative of multiple rearrangements of the transforming plasmid.

#### *bar* Gene Expression in Transformed Plants

One week after the regenerated plants were transferred to soil for acclimation to greenhouse conditions, the leaf tip was



**Figure 4.** PAT activity in protein extracts of leaf tissue. The first lane (NT) contains an extract from a nontransformed control plant. The next seven lanes contain extracts from putative transformants selected for their ability to form roots on bialaphos-containing medium. The sample in the last lane (Mz) serves as a positive control and contains an extract from a maize callus line transformed with a plasmid containing the *bar* gene under control of the cauliflower mosaic virus 35S promoter (R.E. Williams and P.G. Lemaux, unpublished data). The position of acetylated phosphinothricin is marked by the arrow.

sampled for determination of PAT activity (Fig. 4). The level of enzyme activity, as measured by production of the acetylated form of phosphinothricin (arrow), is highest in plants 12D, 9S, and 8W, moderate in 2A, and nondetectable in 6K and 12K. The level of PAT activity is not correlated to the DNA copy number. For example, although line 9S contains high levels of enzyme activity, it has only two *bar*-homologous fragments. Line 6K contains the highest number of copies of the intact ubiquitin:*bar* HindIII fragment (Fig. 3), yet no more than background levels of PAT activity. Yet all these plants clearly exhibited bialaphos resistance at the rooting stage.

#### Inheritance of Bialaphos Resistance Genes

To verify inheritance of the bialaphos resistance phenotype without waiting for the seeds to mature, 38 immature embryos were excised from a control and from a 2A plant 15 DAF and plated on MS agar with or without bialaphos selection. All 19 of both the 2A and control embryos germinated into plantlets with shoots and roots on the medium lacking bialaphos (upper Fig. 2J), showing that the early development of embryos in both the transformed and nontransformed plants was normal. Nine of 19  $T_1$  embryos from line 2A germinated in the presence of 3 mg/L of bialaphos, whereas none of 19 nontransformed embryos germinated on the herbicide (lower Fig. 2J). Six resistant  $T_1$  plantlets were transferred to soil and all exhibited PAT enzyme activity in leaf extracts (data not shown). To determine the segregation ratio of the resistance trait, 49 seedlings were germinated from mature  $T_1$  seeds and sprayed 10 d later with a 2% solution of the herbicide Basta. Forty-two of the seedlings survived. Thus, the bialaphos resistance trait apparently segregated in a 3:1 ratio, indicative of single insertion site for the functional *bar* transgene(s) ( $\chi^2 = 2.2$ ,  $0.2 < P < 0.1$ , with 1 degree of freedom and the Yates correction factor).  $T_1$  plants were further tested for phosphinothricin resistance at the boot stage by application of 2% Basta (Fig. 2K). The

transgenic plant was completely resistant and went on to flower and set seed, whereas the control plant was dead within 2 weeks.

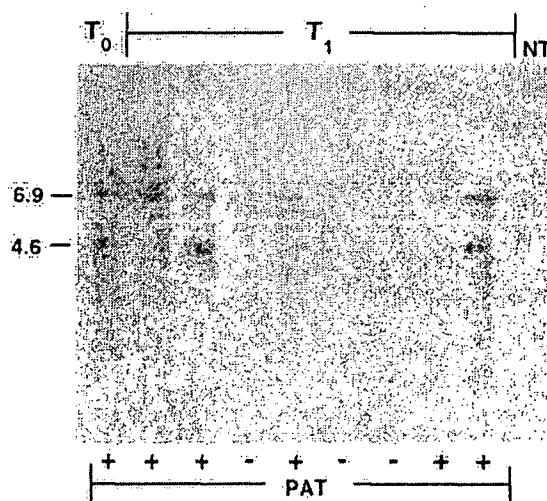
To verify the co-segregation of the resistance phenotype and the transgene(s), DNA was isolated from both herbicide-resistant ( $PAT^+$ ) and -sensitive ( $PAT^-$ )  $T_1$  plants and analyzed for the presence of *bar* sequences (Fig. 5). The  $PAT^+$  plants inherited both *bar*-homologous DNA fragments that were present in the  $T_0$  plant, whereas sensitive plants inherited neither. Thus, in plant 2A, the plasmid DNA is integrated into the wheat nuclear genome at either a single site or at two closely linked sites.

#### Summary of Transformation Experiments

Table I summarizes the results of several bombardment experiments that resulted in production of callus lines or plants shown to contain detectable PAT enzyme activity and/or *bar*-homologous DNA fragments. Independent lines of stably transformed plants were produced at various times of the year, regardless of whether the embryos used for bombardments were obtained from plants grown in the short days of winter, e.g. bombardment 2, or the long days of summer, e.g. bombardment 15. Overall, the frequency of obtaining transformed fertile plants was 1 to 2 per 1000 embryos bombarded.

#### DISCUSSION

As summarized in Table I, we have obtained multiple independent callus lines of transgenic wheat. All but three of these have yielded one or more plants, and all but one of these plant lines were fertile. The procedure used to obtain



**Figure 5.** DNA gel blot analysis of leaf DNA from  $T_0$  and  $T_1$  plants of line 2A. DNAs from the parental plant ( $T_0$ ), eight progeny plants ( $T_1$ ), and a nontransformed control plant (NT) were digested with HindIII and hybridized to *bar*. The sizes in kb of the two *bar*-homologous fragments of parental plant 2A were deduced from other gels run with marker standards and are shown to the left. The results of the PAT enzyme assay for each 2A plant are shown below each lane.

Table 1. Summary of transformation experiments

Bombardment <sup>a</sup>	Date Bombarded	No. of Embryos Bombarded	No. of Transformation Events <sup>b</sup>	No. of Events Producing T <sub>0</sub> Plants	No. of T <sub>0</sub> Plants
2	2-28-92	91	1	1	4
3	3-04-92	68			
4	3-24-92	342			
5	4-03-92	260			
6	4-14-92	139	1	1	1
7	4-20-92	268			
8	5-04-92	613	1	1	2
9	5-11-92	694	3	2	21
10	5-18-92	344			
11	5-26-92	417			
12	6-17-92	1043	6	5	37
14	8-17-92	664			
15	8-31-92	830	1	0	0
16	9-21-92	475			
Total		6248	13	10	65

<sup>a</sup> Bombardment 1 was used for transient expression experiment and bombardment 13 was lost to contamination. <sup>b</sup> As determined by resistance of tissue to bialaphos at the callus and rooting stage and the presence of either PAT enzyme activity or DNA homologous to *bar* coding sequence.

transformants has several advantages over that previously published for wheat. It is reproducible in our hands, yielding 1 to 2 transgenic plant lines per 1000 embryos bombarded. It is relatively rapid. The target tissue takes only 5 d to establish in culture, in contrast to the time-consuming and labor-intensive process needed to establish regeneration-competent cell suspension or callus cultures. On the average, about 5.5 months (168 d) elapsed between excision of embryos for bombardment and anthesis of the transformed T<sub>0</sub> plants. There are two main sources of variation in the time it takes to produce transgenic progeny. One is the season of the year, which determines the length of time for the various growth stages in the greenhouse. The second is the regeneration process. Green sectors arise at variable times after the resistant callus lines are established. The shortest time we have observed thus far between bombardment and the identification of a transformed plantlet in the rooting assay is 75 d.

Our results show that, among the various phenotypic criteria used to assess transformation, the ability of the plantlets to root in the presence of bialaphos was the most reliable indicator of stable integration of the *bar* transgene. Each plant analyzed thus far that exhibited resistance at the rooting stage contains DNA fragments homologous to the *bar* coding region. In contrast, results of the PAT assay were not strictly indicative of transformation and must be interpreted with caution. Whereas a positive PAT assay demonstrated the presence of stably integrated *bar* genes, a lack of PAT activity was not necessarily indicative of the absence of such genes. Protein extracts from nontransformed wheat leaves and calli contain a background activity that acetylates phosphinothricin at a low level (not visible in this reproduction of Fig. 4). Our results indicate that the PAT enzyme assay cannot detect activity below this threshold even though enough enzyme is present in plantlets and callus to confer resistance to phosphinothricin.

Our transgenic plant lines exhibited a range of fertility. All the lines except 6K yielded some seed without outcrossing or embryo rescue, but only 2A had completely normal levels of seed set. It will be interesting to see whether the partial fertility trait is the result of the selection process and thus limited to the T<sub>0</sub> generation or is exhibited by the T<sub>1</sub> progeny of these plants. The latter result would be indicative of an effect of the presence of the transgene on fertility. In the T<sub>0</sub> plants, reduced seed set is not correlated with high expression levels of the transgene, since the sterile line 6K contains no more than background levels of PAT enzyme activity.

This is the third report of the use of the maize *Ubi1* promoter in cereal transformation vectors. Plasmids containing ubiquitin:GUS and ubiquitin:*bar* were used by Cornejo et al. (1992) and Toki et al. (1992), respectively, to identify transformed callus lines in rice. Here we show that this promoter/gene combination can be used to identify wheat transformants. The ubiquitin:*bar* transgenes must be expressed in a wide variety of cell types in wheat, since phosphinothricin resistance is exhibited at several different stages of development: callus tissue, shoot and root regeneration, seedlings, and mature plants. The presence of the ubiquitin:GUS portion of pAHC25 in the genomes of these wheat plants has not yet been assessed. Transgenic plants with functional copies of ubiquitin:GUS could be used to examine in detail the tissue specificity and developmental timing of expression of the maize *Ubi1* promoter in wheat.

Several features of the transformation procedure described in this report will facilitate its adoption by other laboratories. The procedure uses standardized conditions for DNA delivery and the commercially available Biolistics device. Thus, no special apparatus need be constructed to reproduce these results. Bobwhite is a publicly available hard white spring wheat. Excision and culturing of immature embryos requires practice, but no particular tissue culture expertise. Furthermore, the use of this tissue as a target for transformation is

probably applicable to other cultivars that can be reliably regenerated from immature embryo-derived callus. Improved culture methods for some of these cultivars, described recently by Perl et al. (1992), should broaden the applicability of these techniques to even more genotypes. We are currently applying our procedures to transformation of a hard red winter wheat cultivar.

The efficiency and reliability of these procedures makes feasible, for the first time, studies of promoter and protein function in transgenic wheat. Transformation methodology will also allow a biotechnological approach to modification of important traits of wheat, including pathogen resistance and bread-making quality.

#### ACKNOWLEDGMENTS

We thank K. Koshiyama for excellent technical assistance, and Dr. P. Lemaux, R. Williams, and Dr. Y. Wan for helpful discussions, encouragement, and assistance with the particle bombardment device. Dr. F. Greene laid the groundwork for this study while he was in our laboratory. Dr. M.-J. Cornejo demonstrated the utility of the maize *Ubi1* promoter in rice transformation studies in our laboratory. We thank Dr. A. Christensen and Dr. P. Quail for pAHC25 and S. Gertula for DNA hybridization conditions applicable to large genomes. We thank Dr. S. Altenbach, Dr. J. Bernardin, Dr. J. Dvorak, and Dr. D. Siler for critical reading of the manuscript. Bialaphos was provided through the generosity of Meiji Seika Kasha, Ltd., and Basta was provided by Hoechst AG.

Received March 15, 1993; accepted April 28, 1993.

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## **EXHIBIT F**

# PLANT PHYSIOLOGY

THIRD EDITION

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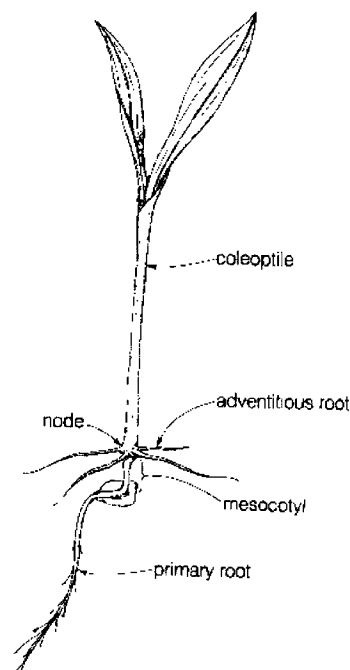
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kept dark or given far-red (Nabors and Lang, 1962). Our conclusion is that  $P_{fr}$  increases the growth of the radicle cells, presumably those in the elongation region, by decreasing their water potential so that they more easily absorb water from soils and germinate. These facts suggest that germination of light-requiring seeds fails in darkness because the radicle does not grow with sufficient force to break through layers that surround it. Of these layers, the lettuce radicle is restricted almost entirely by the tough endosperm, even though it is only two or three cells thick. The endosperm is also the restrictive layer in *Phacelia tanacetifolia* and various *Syringa* (lilac) species. For lettuce, only increased thrust of the radicle seems important, even though the endosperm is weakened greatly after germination is well underway (Bewley and Black, 1982). For other seeds, one might reasonably expect  $P_{fr}$  to increase germination either by increasing radicle thrust or by weakening surrounding barriers to its growth, or both. Seed dormancy is less a mystery when we consider germination as a struggle between the growth potential of the radicle and the growth-restrictive mechanical effects of surrounding layers. In some cases, the mechanical restriction is great; in others it is of little consequence, and only a small increase in the radicle growth potential caused by  $P_{fr}$  is enough to cause germination. Nevertheless, even in these, the reduction of  $P_{fr}$  by far-red light reduces germination.

**Effects of Hormones on Photodormancy** In most dormant seeds, applied gibberellins substitute for the light requirement; and for a few species such as lettuce, cytokinins also substitute for light or partially replace it. Auxins usually do not promote germination of photodormant or nondormant seeds and are instead either innocuous at low concentrations or inhibitory at high concentrations. The role of ethylene is less clear. It cannot break photodormancy, but can partially overcome other kinds of seed dormancy in cocklebur and in certain peanut and soybean varieties. It can also partially overcome dormancy caused by high temperatures in lettuce and partially overcome photodormancy problems in cocklebur (Bewley et al., 1983). Abscissic acid almost always inhibits germination, because of its growth inhibitory effects.

Collectively, these results suggest that  $P_{fr}$  might break photodormancy by causing synthesis of a gibberellin or a cytokinin or by destroying an inhibitor such as ABA. The evidence about this is presently controversial (Bewley and Black, 1982; DeGreef and Bewley, 1983), but no one has yet measured hormone changes only in the radicle or hypocotyl cells responsible for germination. This seems essential to understand relations among light, growth hormones, and growth inhibitors in photodormancy.



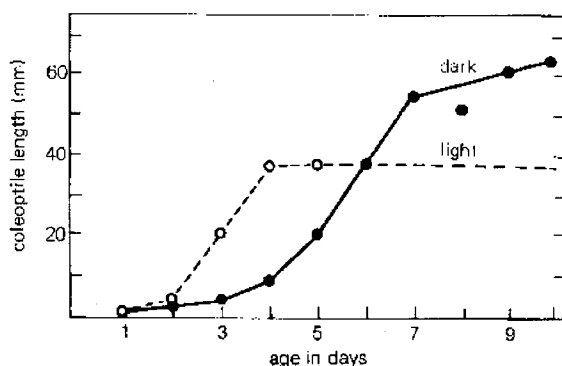
**Figure 19-7** Some morphological characteristics of a week-old maize seedling grown in light. The coleoptile has stopped elongating, and two leaves have broken through it and have largely unrolled. The shoot apex is at the node where adventitious (prop) roots originate. The mesocotyl is the first internode formed above the seed storage tissues and the scutellum (cotyledon) in the seed.

and other kinds of seed dormancy discussed in Chapter 21. Analyses of whole seeds for hormone levels seem nearly useless in understanding hormonal aspects of dormancy, because the whole seed is so large relative to the few tissues that control germination.

### 19.5 The Role of Light in Seedling Establishment and Later Vegetative Growth

Once germination is accomplished, further plant development still remains subject to control by light. We introduced some of these controls in Section 19.1 and Fig. 19-1. We now evaluate these and other effects and ask whether phytochrome is the only pigment involved and how it acts.

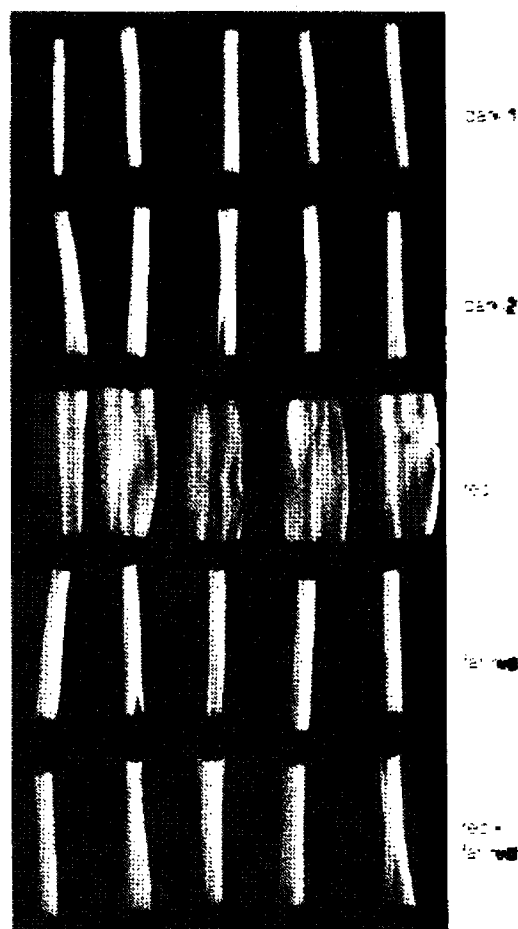
**Development of Poaceae Seedlings** After a grass or cereal grain seed germinates, its coleoptile elongates until the tip breaks through the soil. Between the scutellum (see Fig. 16-12) and the base of the coleoptile is an internode called the **mesocotyl** (first internode, Fig. 19-7) that in some species elongates greatly



**Figure 19-8** Elongation of oat coleoptiles in darkness and in continuous white light. Light at first promotes growth but later is inhibitory. (From B. Thomson, 1954.)

after germination of deeply planted seeds. Elongation of the mesocotyl, coleoptile, and leaves enclosed by the coleoptile is necessary to carry leaves into the light and to establish near the soil surface the adventitious roots produced at the node just above the mesocotyl (Fig. 19-7). Elongation of the mesocotyl has received attention for more than 40 years. All results show that mesocotyl elongation is extremely sensitive to light (Mandoli and Briggs, 1981; Schäfer et al., 1982). In oats, growth of the mesocotyl is slowed over a 54-hour period by continuous red light at photon irradiance levels of only  $5 \times 10^{-16}$  moles of photons per square meter of tissue per second (Schäfer et al., 1982). This irradiance level is only about  $10^{-7}$  that in all visible wavelengths provided by moonlight. This effect of light on plants is by far the most sensitive one known, and no green safelights are safe in studies with this response.

Elongation of the coleoptile must equal or exceed that of the leaves it encloses as they grow upward together; otherwise the leaves would grow out of the coleoptile and probably be broken off in the soil. Growth rates of these two organs are coordinated until they reach the soil surface and are exposed to light. After exposure to light, the leaves become green and photosynthetic, and they break through the coleoptile tip. Leaf emergence occurs because light promotes leaf elongation and decreases the extent to which coleoptiles can elongate (although it speeds their early elongation) (Schopfer et al., 1982). Furthermore, breakage of the coleoptile tip by the elongating first leaf stops coleoptile elongation, presumably because this stops auxin transport from the tip to elongating cells below. The light promotion of leaf growth and inhibition of coleoptile growth are phytochrome responses of sunlight. Figure 19-8 shows that the overall effect of continuous light is to reduce coleoptile elongation, even though during the first few days elongation is promoted. The reasonable conclusion from these 1954 results is that light has-



**Figure 19-9** Effect of pretreatment with red and far-red light on unrolling of leaf sections from etiolated maize seeds. Red promotes opening, whereas subsequent far-red nullifies the red effect. (From W. H. Klein, L. Price, and K. Mitrakas, 1963.)

tens growth and maturation of young coleoptiles because those cells begin to elongate earlier and mature earlier than in darkness. Effects of light on elongating cells reaching maturity show that elongation is slowed and that maturation occurs earlier in darkness. More recent results verify those early conclusions and further show that phytochrome is the controlling photoreceptor of light, even though a term light exposure that results in high irradiance values is necessary to cause the responses (Schäfer et al., 1982).

Figure 19-7 illustrates a maize seedling grown in light from a seed planted near the soil surface. The mesocotyl had elongated very little, and the first leaves had emerged from the coleoptile. Each leaf was rolled up inside the coleoptile, but when exposed to light they began to unroll (flatten). Rolling was still evident only at the point of

parture from the broken coleoptile. Unrolling of grass leaves is controlled by a typical phytochrome response, low irradiance levels of red promoting and subsequent far-red nullifying the red effect (Fig. 19-9). Low energies of far-red are without effect, and low energy blue is only slightly promotive except in rice. Unrolling is caused by more rapid growth of cells on the concave (to be uppermost) than the convex side. Whether this growth is caused by wall loosening, solute production that decreases the cells' osmotic potential, or both, is not known. Nevertheless, exogenous gibberellins and, to a lesser extent, cytokinins replace the need for light and  $P_{fr}$  (DeGreef and Fredericq, 1983). These results suggest that  $P_{fr}$  causes rolled leaves to form gibberellins or cytokinins which then cause unrolling. This hypothesis might be correct for gibberellins, because  $P_{fr}$  promotes gibberellin production and release from young plastids in rolled wheat and barley leaves much sooner than the leaves unroll. No studies showing light effects on cytokinin contents of unrolling leaves are available, so for now it seems safest to conclude that light might induce leaf unrolling by causing production of gibberellins in concave cells. Alternatively, the concave cells might become more sensitive to the hormone levels they already contain when exposed to light (see the personal essay by A. Trewavas in Chapter 16).

**Development of Dicot Seedlings** In dicots, the cotyledons either remain underground by **hypogeal development**, as in pea, or emerge above ground **epigeally**, as in beans, radish, and lettuce. In either case, a hook is formed near the stem apex that pushes through the soil and pulls with it the fragile young leaves or cotyledons. (In Fig. 19-1 this hook has moved, as it does in seedlings that develop epigeally, to the epicotyl (stem section above cotyledons) and has opened somewhat, perhaps by slight light exposure during watering.) As mentioned in Section 15.2, this hook forms as a result of unequal growth on the two sides of the hypocotyl or epicotyl in response to ethylene soon after germination. As the hook emerges from the soil, red light acting through  $P_{fr}$  promotes opening of the hook. Hook opening apparently results from inhibition by light of ethylene synthesis in the hook. Differential growth that results in faster elongation of cells on the lower (concave) side than on the upper (convex) side causes hook opening (Section 15.3). Accompanying this, light increases leaf blade expansion, petiole elongation, chlorophyll formation, and chloroplast development, as in grass leaves (Fig. 19-1), and  $P_{fr}$  also speeds stem elongation.

Most of the light promotion of leaf growth, at least in dicots, is caused by an HIR (Dale, 1982). A good example is provided by the primary leaves of

bean. Plants grown 10 days under dim red light have slightly larger leaves and substantially more cells than those kept in darkness; but when they are transferred to white light, cell expansion and leaf growth increase greatly. In this case the HIR system causes expansion by enhancing acidification of the cell walls, thus loosening them so that they grow faster under turgor pressure (Van Volkenburgh and Cleland, 1981).

Light effects on chlorophyll formation and chloroplast development result first from a triggering action of  $P_{fr}$  that causes production of **delta-aminolevulinic acid (ALA)**, probably from glutamic acid (Kasemir, 1983; Castelfranco and Beale, 1983). ALA is the metabolic precursor converted into each of the four pyrrole rings of chlorophyll. Nevertheless, ALA is not converted all the way to chlorophyll without higher irradiance red or blue light. Instead, the metabolic pathway stops when a compound often called **protochlorophyll** is formed. More accurately, protochlorophyll is **protochlorophyllide *a***, which differs from chlorophyll *a* (Fig. 9-4) only by the absence of a phytol tail and two H atoms. Protochlorophyllide *a* is rapidly reduced to chlorophyllide *a* in red or blue light, because protochlorophyllide *a*, like chlorophylls, absorbs those photons effectively. Addition of the phytol tail, an isoprenoid formed from the mevalonic acid pathway (Chapter 14), completes formation of chlorophyll *a*; some of the chlorophyll *a* is then converted to chlorophyll *b*. Chloroplast development depends strongly on chlorophyll formation and, therefore, on both of these light effects, although there are other light responses that we shall not discuss (Kasemir, 1983; Virgin and Egnéus, 1983). All of these responses lead, within a few hours, to photosynthesis in grass leaves as they break through the coleoptile and in cotyledons or young leaves of dicots as they break through the soil. Cotyledons of conifers somehow form chlorophyll and become photosynthetic even in darkness, but their needles require light for these processes.

As photosynthesis begins in leaves and cotyledons, stem elongation is inhibited by light. Of course, the seedling cannot elongate after its food supplies are exhausted; but while carbohydrates or fats are still plentiful, light is inhibitory. This inhibition of stem elongation was apparently first recorded by Julius von Sachs in 1852. He observed that stems of many species do not grow as fast during daylight as they do at night. We now realize that blue, red, and far-red all contribute to this phenomenon and that cryptochrome and phytochrome are both responsible. Hans Mohr and others in Germany worked extensively with etiolated seedlings of white mustard (*Sinapis alba*) and measured many responses in these, which are summarized in Table 19-1. They also measured the action spectrum for inhibition of